PHOTOSYNTHETIC MECHANISMS OF GREEN PLANTS

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Chairman: Bessel Kok
Organizer: André T. Jagendorf

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FOREWORD

This volume contains the papers submitted to a symposium on "Photosynthetic Mechanisms of Green Plants," held at Airlie House, Warrenton, Virginia, Oct. 14-18, 1963. The symposium was proposed and sponsored by the Committee on Photobiology, of the National Academy of Sciences—National Research Council, Carl P. Swanson, Chairman. The funds for the symposium were provided by a generous grant from the National Science Foundation, with ancillary support from the Kettering Foundation, Yellow Springs, Ohio.

It was the feeling of the Committee that progress in the field since the last meeting of this kind, Gatlinburg, 1955, warranted a summing up and organization of our newer information. In retrospect, a brief paper by Blinks in that last meeting proved to be the forerunner of the period in photosynthesis research which we attempted to crystallize at Airlie. Blinks' report on chromatic transients brought the realization that photosynthesis is not "color blind"—and that different pigments might sensitize different photoprocesses. Soon followed the observation by the late Dr. Emerson of the enhancement effect in which lights of two different wavelengths proved to exert a greater effect if given simultaneously than if given individually. This enhancement of net rate was rationalized by the observation of a push-and-pull effect of two different colors upon intermediate catalysts of the process: i.e., P700 and cytochrome f. The analysis of photosynthesis in terms of two distinct photoreactions, their features and their coupling, has consequently been the main area of concentration during the last years. This interest is reflected in a large percentage of the papers in this symposium. Enhancement, transients, and respiratory interactions with at least one light reaction have been studied in great detail. Considerable spectroscopic evidence concerning primary and early events has accumulated. Also on the basis of biochemical studies and analysis using mutants, a picture of photosynthetic electron transport is beginning to emerge, albeit somewhat hesitantly. There seems to be fair agreement at present about the nature of the first photoreaction, producing an as yet unidentified strong reductant, and a weak oxidant (P700). Compared to this the details of the other photoact and the evolution of oxygen associated with it are still quite obscure.

Considerable progress is reported in regard to the nature and function of known and newly discovered constituents of the electron transport chain: chloroplast ferredoxin, transhydrogenase, plastoquinones, cytochrome f, and plastocyanine. Photophosphorylation in chloroplasts, discovered about the time of the previous meeting in Gatlinburg, appears to be far from a finished problem. Some newer aspects are presented here, such as a large pool of high-energy intermediate, capable of making ATP in the dark.

It is interesting to note that probably no area of photosynthesis can yet be considered a closed chapter. Although the path of carbon in photosynthesis

has been familiar for a considerable time, the present fluid state of this field is excellently surveyed, with clear indications as to where revisions must be forthcoming in the future. Elegant work on the chemical structure of lamellae points up our continued ignorance as to the precise functional significance of the structures, or even as to the mode of attachment of chlorophyll to protein.

Our introduction cannot attempt a detailed survey of the contents of this book. It must further be stated that some areas of photosynthesis were completely omitted in the planning of this meeting. We hope the reader realizes that the constant shifting of the focus of attention often makes it difficult to discriminate between the intrinsic importance of a given aspect and the number of workers interested in it. Every so often someone manages to remove another stone from the wall through which we all want to see, and the crowds tend to flock around the new peep-hole.

To a certain extent we must apologize for the inelegant composition of this volume, the lack of editing and the somewhat brutal measures taken to secure early publication. We are grateful to all contributors for their cooperation in providing concise summaries of their recent important work, and we hope that the exposed cross-section of photosynthesis as of the early sixties will prove of value.

It is a pleasure to acknowledge the conscientious efforts of Miss Inger Hermann, Secretary of the Photobiology Committee, and of her staff.

Bessel Kok, Chairman

André Jagendorf, Organizer

I. SPECTROSCOPIC AND FLUORESCENCE ANALYSIS OF OXIDATION-REDUCTION CATALYSTS

STUDIES ON PRIMARY REACTIONS AND HYDROGEN OR ELECTRON TRANSPORT IN PHOTOSYNTHESIS BY MEANS OF ABSORPTION AND FLUORESCENCE DIFFERENCE SPECTROPHOTOMETRY OF INTACT CELLS

L.N.M. Duysens

INTRODUCTION AND METHODS

As requested by the organizers of this symposium, in this paper investigations mainly carried out at our laboratory will be reviewed and interpreted.

In most experiments the time courses of changes in light absorption and emission were recorded at various wavelengths in the visible and adjacent spectral regions for suspensions of intact photosynthesizing cells. These changes were brought about by suddenly admitting relatively strong so-called actinic light of constant intensity. This light was switched off after a time varying from one second to about one minute. The changes in absorption were measured by means of a separate weak modulated measuring beam, which in general did not cause changes in absorption[1,2]; the fluorescence was in general excited by modulated light of such a low intensity so as not to cause changes in fluorescence yield[3]. The measuring apparatus was only sensitive to the modulated measuring beam or to the fluorescence, excited by the modulated exciting beam. These modulations and the use of suitable filters prevented direct effects of the actinic light on the measuring apparatus. The absorption and fluorescence changes were recorded by means of a one-fourth or one second recorder. Rapid changes in fluorescence yield were brought about by an electronic flash, and were measured by means of an oscillograph. This apparatus has a response time of the order of one μsec, and the flash served both as exciting and actinic beam.

A difference spectrum presents a change in the absorption spectrum or of the fluorescence spectrum occurring during illumination[1]. Difference spectra may be obtained from the time curves of absorbancy or fluorescence changes (see[1]). These spectra sometimes permit the identification of substances causing the spectral changes. If e.g. the difference spectrum is

equal to the difference of the absorption spectra of oxidized and reduced cytochrome, this shows that upon illumination a cytochrome becomes oxidized. Similarly, if the fluorescence difference spectrum is equal to the fluorescence spectrum of chlorophyll a, this means that the chlorophyll a fluorescence increases upon illumination. Difference spectra determined between various time limits make it possible in principle to analyze the time course of photosynthetic intermediates which show absorption or fluorescence changes upon illumination.

By measuring the effectivity of the actinic light as a function of the wavelength, it is possible in principle to identify the pigments which are responsible for the photochemical reaction or reactions which cause changes in the absorption spectrum or the fluorescence spectrum. If these changes are qualitatively different in light of different actinic wavelengths, then it follows that more than one photochemical reaction driven by pigment systems with different absorption spectra occurs. This is the case in oxygen evolving photosynthesizing organisms. In fluorescence experiments one more parameter is available: the wavelength of the exciting light. The action or effectiveness spectrum for exciting the fluorescence of a certain compound is proportional to the sum of the absorption spectra of the fluorescing substance and of the absorption spectra of other pigments multiplied by the efficiences of transfer of excitation energy from these pigments to the fluorescing substance[4,5].

Since it is possible to bring about oxidation or reduction of a great number of non-physiological substances by means of extracts of bacteria or higher plants, it is in general not possible to conclude from these experiments, whether a certain redox reaction occurs in the living cell, even if the reacting substances are known to occur in the cell. For this reason mainly, we have concentrated on studying reactions in intact cells in order to establish which reactions occur in vivo. Most if not all reactions observed by absorption and fluorescence techniques in living cells were redox reactions. In order to find out whether a reaction is a main pathway in photosynthesis or a side reaction, the quantum efficiency of this reaction may be determined. Since the quantum requirement per hydrogen atom or electron transported for CO_2 reduction is 2, an efficiency of the same order of magnitude may be expected for the redox reactions in the photosynthetic chain.

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Qualitative evidence suggests a hydrogen transport scheme with two different photochemical reactions in series. If the two pigment systems have different pigment compositions, in absence of interaction two different action spectra for the various reactions are found under the appropriate conditions. We feel that quantitative data, such as quantum efficiences and action spectra for the various reactions, are very useful perhaps necessary criteria to establish whether a hydrogen transport scheme may be correct.

RESULTS AND INTERPRETATION

For the sake of brevity the results will not be discussed in a historical and inductive sequence, but rather in a logical and deductive one. The mechanism of hydrogen or electron transport in algae or higher plants is discussed at the hand of the scheme shown in Fig. 1.
The arrows point into the direction of hydrogen transport. Of the two components of the redox couples, only the component preponderant in darkness is represented. Cytochrome C 420 occurs in darkness in the reduced form. The approximate E'_0 values of the redox couples are indicated at the left side: the more strongly reducing redox couples occur at the top of the drawing. The two heavy upward pointing arrows indicate the two primary photochemical reactions, 2 and 1, symbolized by the light quanta $h\nu_2$ and $h\nu_1$. Most other arrows point downward, which indicates that the reactions occur spontaneously with a loss of free energy. Such reactions may be "coupled" to a reaction in which a gain of free energy occurs: the phosphorylation of ADP. The two primary photochemical reactions, photoreactions 1 and 2, are driven by two distinct pigment systems with different absorption and action spectra. Action spectra of these systems are shown for two species as inserts in the scheme. The action spectrum of pigment system 1 in the red alga Porphyridium cruentum shows that for photoreaction 1 quanta incident at 680, which are mainly absorbed by chlorophyll a, are about equally effective as quanta absorbed at 560 mμ mainly by the "accessory pigment" phycoerythrin . The spectrum of system 2 shows that quanta incident at 560 mμ, which are absorbed mainly by the phycoerythrin of pigment system 2, are much more effective in photoreaction 2 than quanta incident at 680 or 430 mμ, which are absorbed by chlorophyll a . Comparison of the action spectra of the two systems shows that light absorbed by Porphyridium at 680 and 430 mμ is more effective in exciting pigment system 1 than system 2, and that light absorbed at 560 mμ is more ef-

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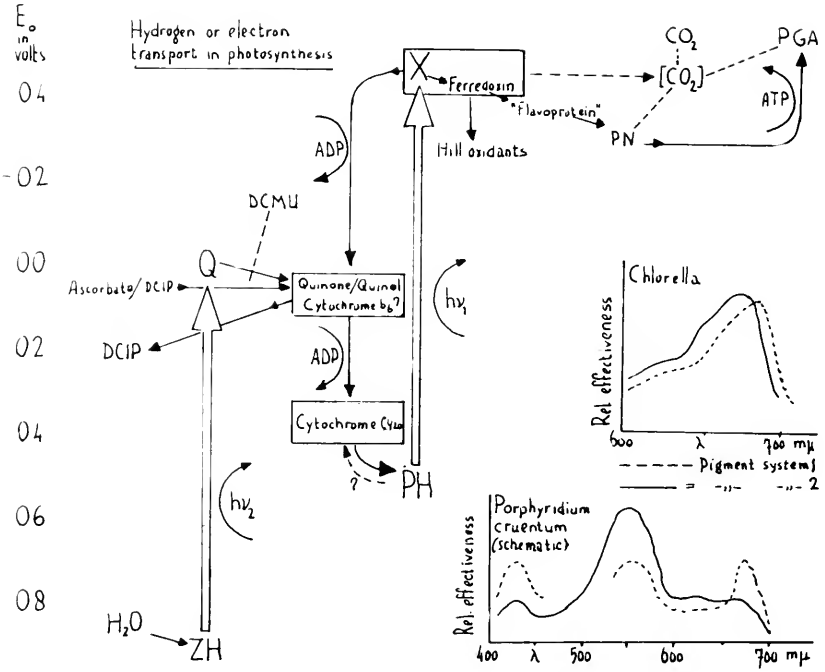

Fig. 1. Scheme for photosynthesis. The direction of hydrogen or electron transport of oxygen evolving photosynthetic organisms is indicated by the direction of the arrows. The redox substances surrounded by a rectangle may be partly bypassed. The two light reactions are represented by the large open arrows. Action spectra of the two pigment systems driving these reactions are shown for two algal species. A more detailed description is given in the text.

fective in exciting system 2. In Chlorella, chlorophyll b is more active in system 2. Both systems contain about equal amounts of chlorophyll a, but a form of chlorophyll a with absorption at longer wavelengths is preponderant in system 1. Wavelengths shorter than 680 mμ, are more effective for system 2 than for system 1 in Chlorella. The relative amounts and activities of the various pigments being present in the two systems is not only different for different species, but also for different cultures of the same species.

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Evidence for the occurrence of the redox couples shown in Fig. 1 will be discussed below. We restrict ourselves here to a few general statements. According to the scheme, upon onset of illumination a reduction will occur of all compounds at the right hand side at the top of the scheme, and an oxidation of H_2O and ZH. Upon illumination with light of a wavelength, which is mainly absorbed by system 1 (and which we call light 1, such as 430 and 680 mμ in Porphyridium, or 700 mμ in Chlorella), redox components in between the two systems, such as Q and PH, tend to accumulate in the oxidized state; in light 2 (such as light of 560 mμ in Porphyridium and of wavelength shorter than 680 mμ in Chlorella) these redox couples may be expected to accumulate in the reduced state. Upon darkening, the initial redox state in the dark is usually reestablished due to reactions with redox substances shown in the scheme, or with other cell constituents. The action spectrum for the initial rate of reduction of X and all compounds written at the right hand side of Xwill be proportional to the action spectrum of system 1; the same will be true for the initial rate of oxidation of the substances between system 1 and 2, with the exception of Q, if the electron or hydrogen transport between Q and the substances is interrupted by an inhibitor such as DCMU. In the presence of this inhibitor, the action spectrum for the initial rate of reduction of Q will be proportional to the action spectrum of system 2. The overall electron or hydrogen transport during steady state photosynthesis will be limited by that system which has the smallest activity at the wavelength of the actinic light. Thus, the action spectrum of photosynthesis will follow the lower of two curves shown in the inserts of Fig. 1. If, on the other hand, the action spectrum of photosynthesis is measured in the presence of a strong constant background of light, mainly absorbed by system 2, this action spectrum will be proportional to the action spectrum of system 1 [6], and vice versa. This was in fact the way French and Myers [7] measured action spectra of Chlorella, and we have interpreted these spectra as action spectra of system 1 and 2. The spectra for Porphyridium, also shown in Fig. 1, were measured in various ways (see below).

The scheme of Fig. 1 makes it possible to understand the " Emerson effect ". Emerson's discovery [8] stimulated research that established and identified the two algal photosynthetic systems. This effect may be formulated and "explained" as follows (cf. [6]). If light beams 1 and 2 (e.g. light of 700 and 650 mμ respectively in Chlorella, or 680 and 560 mμ in Porphyridium) are applied simultaneously, then the rate of photosyn-

thesis in both beams applied together is found to be greater than the sum of the rates in each beam separately. In light 1, system 1 is producing oxidized intermediates at a higher rate than system 2, and in light 2, system 2 is producing reduced intermediates at the higher rate. If both beams are applied simultaneously, the oxidized and reduced intermediates formed in excessin each beam separately react with each other, which results in an enhanced electron transport or an enhanced rate of photosynthesis.

Cytochrome reactions

Purple bacteria. Illumination of purple bacteria causes the oxidation of one or more cytochromes[9,10,11,12,13]. In purple bacteria presumably only one photochemical system, analogous but not identical to system 1 in algae and higher plants is present[14,15]. Oxidized cytochrome may be reduced either by a so-called hydrogen donor, which has a function analogous to H_2O in algae, or by a reductant, e.g. by XH, formed in the light. In the latter reaction so-called cyclic phosphorylation may occur[16,17]. The sequence of the reactions in purple bacteria between the various cytochromes present, has not yet been definitely established. Olson and Chance[12] made an extensive investigation of cytochromes in the purple bacteria Chromatium. They concluded that at least four cytochromes were present, distinguished by different time courses and difference spectra. One of these cytochromes, C 423,5 (with a difference spectrum having a soret maximum at 423.5 mμ) is still oxidized at the temperature of liquid air[18]. Oxidation of other cytochromes was not observed at this temperature, but it was suggested that the oxidation of these cytochromes could not be observed, because these cytochromes were oxidized spontaneously in darkness during cooling.

The rate of oxidation of C 423.5 was the same at -196°as at 20°C. Cytochrome photooxidation is an extremely efficient reaction. Assuming that the specific absorption difference at 420 mμ is 62 /(mM·cm), Olson[19] calculated that the number of quanta required for the oxidation of one cytochrome molecule (the quantum requirement) was about 1 within rather large limits of error. Making similar assumptions, Vredenberg[20] calculated a minimum requirement of 0.6, which suggests that the true quantum requirement is 1 and that the true specific absorbancy difference at 423 mμ is about 100 /(mM·cm). The equality of the rates (and thus that of the quantum requirementsof cytochrome oxidation) at 20° and at -170°C was confirmed. However, it was found that

the rate (or the quantum requirement) decreased at -220°C. Also it was established that all cytochromes, which were at 20°C in the reduced state, remained in the reduced state during cooling in the dark; except for C 423.5, no photooxidation occurred at -170°C. The rate of photooxidation of cytochrome C 422 strongly decreased for temperatures below -90°C (see Fig. 2).

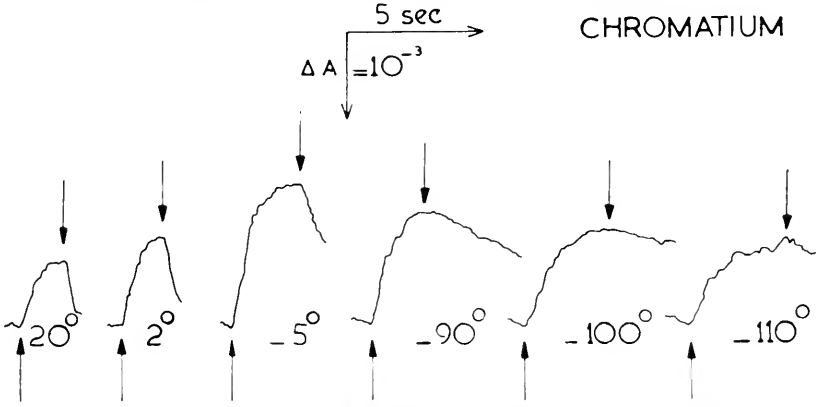

Fig. 2. Time courses showing photooxidation of cytochrome C 422 in Chromatium (28) at various temperatures but for the same intensities. The initial rate of oxidation decreases below -90°C.

The decrease in the rate upon lowering the temperature was relatively more pronounced at higher intensities of the actinic light, which also indicated that the rate was limited by a temperature dependent reaction. The photooxidation of C 422 (above -90°C) was observed under conditions, in which C 423.5 was oxidized, and was found to proceed with a quantum requirement of 1, if the specific absorbancy difference is assumed to be 100/(mM·cm). These dark redox reaction may differ from other dark reactions which stop at temperatures close to the freezing point in that the participating molecules do not have to diffuse before reacting. These observations suggest that C 423.5 and C 422 are located on the same protein or cell constituent, and that oxidation of C 423.5 causes a change in confirmation of this protein, which brings the haem moiety of C 422 in the right position to be photooxidized even at a temperature of -100°C by the probably adjacent primary photooxidant, P 890 (see below).

Cytochrome reactions in algae. The absorption difference spectrum, light minus dark, of the red alga Porphyridium cruen-

tum, which has peaks at 420 and 555 mμ, is similar but not identical to the difference of the absorption spectra of oxidized and reduced cytochrome f or c[23]. We call this cytochrome C 420. The maximum of the difference spectrum of cytochrome f in vitro occurs at a 424 mμ[21,23]; the band shift may have been caused by the extraction procedure. In contradistinction to the action spectrum of photosynthesis[5][2], which shows much stronger photosynthetic activity of phycoerythrin than of chlorophyll a, the action spectrum for cytochrome oxidation[2] shows strong activity for chlorophyll a but little activity for phycoerythrin; furthermore, the kinetics of cytochrome oxidation were qualitatively different at 680 and 560 mμ, which indicated that at least two photochemical reactions participated in the cytochrome reactions. If the cytochrome is brought in the oxidized state by illumination with actinic light of 680 mμ, addition of strong light of 560 mμ caused a reduction of the cytochrome[15,6]. As discussed in the introduction, oxidation by light 1 and reduction by light 2 can be explained by means of scheme 1, and, in fact, a scheme like this was first proposed on basis of the cytochrome reactions. The light-driven reduction of cytochrome was inhibited by DCMU, hydroxylamine or N-ethylurethane. This suggests that these inhibitors inhibit one or more reactions between photoreaction 2 and C 420. Evidence will be given below that DCMU inhibits between Q and quinone. From scheme 1 it follows that in the presence of an inhibitor like DCMU the action spectrum for the initial rate of C 420 oxidation is proportional to the action spectrum of system 1. The action spectrum of system 1 of Porphyridium between 540 and 710 mμ shown in the insert of the scheme was determined in this way. The action spectrum of photosynthesis, measured against a background of relatively strong light of 560 mμ is, according to the discussion in the introduction, also proportional to the action spectrum of system 1, and was found to be similar to the action spectrum for cytochrome oxidation, albeit not identical. So far only ad hoc explanations have been given of the deviation between the two spectra[6]. Similar observations were made for the blue-green alga Anacystis nidulans[24].

Phosphopyridine nucleotide reduction. Upon illumination of photosynthetic organisms an increase in fluorescence in general occurs in the blue region. The fluorescence difference spectrum and that for the excitation of this fluorescence are similar to the corresponding spectra of PNH bound to an enzyme. Since DPN and TPN do not fluoresce, these experiments indicate that upon illumination of photosynthetic organisms reduction of py-

ridine nucleotide occurs[25].

In algae, pyridine nucleotide reduction could be most readily studied in the blue-green alga Anacystis nidulans[24]. As predicted by the scheme of Fig. 1, the action spectrum for the initial rate of pyridine nucleotide reduction was found to be roughly proportional to the action spectrum for C 420 oxidation in the presence of an inhibitor of the cytochrome reducing reaction. Measurements of the quantum requirement for this reduction were consistent with the assumption that one hydrogen absorbed by system 1 was sufficient for the transport of one hydrogen-equivalent to pyridine nucleotide. Since about 8 quanta (absorbed by both pigment systems) are probably necessary for the reduction of 1 CO_2 molecule [26], this implies that each photochemical system requires one quantum per transported hydrogen atom or electron. The quantum requirement for C 420 oxidation by system 1 is estimated to be higher than 2 for Porphyridium [24,6] and still higher for Anacystis [24], which suggests that part of the hydrogens transported from $Q\bar{H}$ to P 700 bypasses C 420. For this reason we have put C 420 within a rectangle. Vredenberg (unpublished observations) observed a much higher efficiency for cytochrome oxidation after cooling to 2°C.

Quinone reactions

Addition of plastoquinone stimulates the Hill reaction under certain conditions as first shown by Bishop[27]. This has been taken as a proof of participation of plastoquinone as a redox intermediate in the Hill reaction. It is also conceivable, however, that the quinone acts as a structural factor, which is necessary for optimal rate of the Hill reaction. Also the observation that quinones are reduced or oxidized by chloroplasts does not prove, as was argued in a general way in the introduction, that quinone is a photosynthetic intermediate. Recently Amesz[28] obtained, upon illumination of the blue-green alga Anacystis, in the ultraviolet region a difference spectrum which was similar to the difference spectrum of oxidized minus reduced plastoquinone (see Fig. 3). Fig. 3 then indicates that light absorbed by system 1 causes oxidation of quinone, and that light absorbed by system 2 favors its reduction. The reduction of quinone was inhibited by low concentrations of DCMU. The time courses of the oxidation and reduction indicate thatthe quantum efficiency for these reactions is rather high. The $E\delta$ value of quinone, and the fact that DCMU inhibits quinone reduction indicates that quinone is located between Q and C 420. The amount of quinone participating in this light-driven redox reaction is only about 1% of the amount of chlorophyll a present. This is

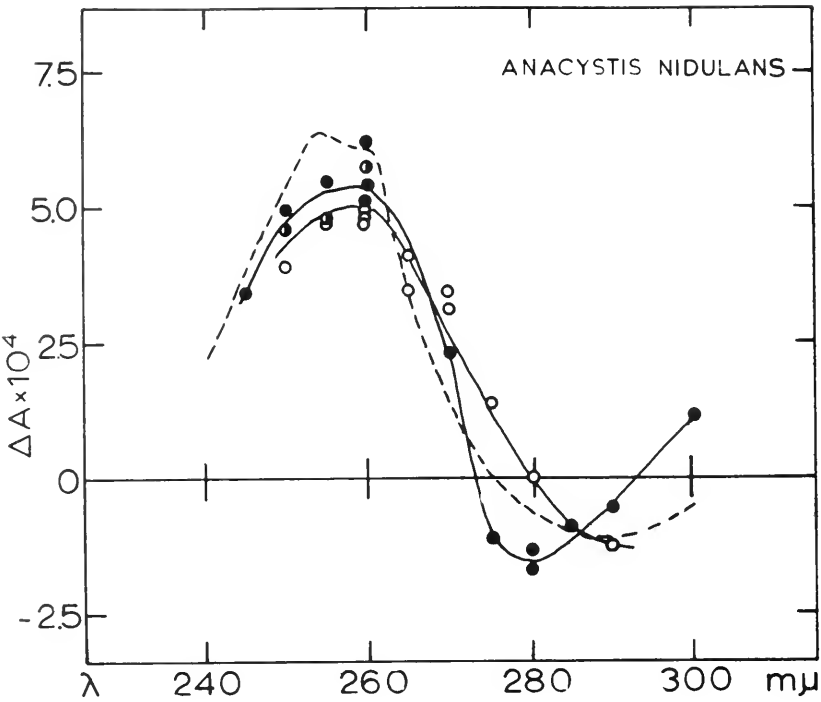

Fig. 3. The difference spectrum with the black circles is the absorbancy difference of the steady states in intense light of 680 mμ (light 1) and in light of 620 mμ (light 2), and that with open circles is the absorbancy difference at lower intensities, between the steady states in light of 680 and 620 mμ and in light of 620 mμ alone. The broken curve is the difference of the spectra of oxidized and reduced plastoquinone. The first spectrum indicates, that besides oxidation of quinone in light of 680 mμ other reactions occur.

only a small fraction of the total amount of the quinones, which are present in the chloroplast(at a concentration of about 1/10 of that of chlorophyll).It was not possible to conclude from the difference spectrum, which of the numerous quinones discovered in chloroplasts is the photoactive one.

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The primary photooxidants of system 1: P 890 and P 700

The first observed reversible change in absorbancy upon illumination of photosynthesizing organisms was a decrease in absorption close to the bacteriochlorophyll peak at around 890 mμ in purple bacteria[5,29]. In various species studied, this decrease was accompanied by an decrease at about 810 mμ and an increase of absorption at about 790 mμ. These absorption changes were attributed to the oxidation of a small(about 2%) special fraction of the bacteriochlorophyll, which fraction we now call PH 890. Since the maximum of the difference spectrum of PH 890 varies in different purple bacteria[5,20] and under various conditions[20] in a similar way as the maximum of the absorption spectrum of the bacteriochlorophyll-type B 890 (see the next paragraph), PH 890 is probably closely related to bacteriochlorophyll, but not necessarily chemically identical. The shape of the difference spectrum indicates that PH 890 consists of at least two bacteriochlorophyll-like molecules, one with a maximum at about 810, the other with a maximum between 870 and 890 mμ, depending upon the species. Upon photoconversion of PH 890 to P 890 the maxima shift from about 810 and 890 mμ to about 790 and 1250 mμ. The last maximum was observed by Clayton[30]. Similar changes in the infrared region can be brought about by adding mixtures of potassium ferro- and ferricyanide. The E'_o value of P was found to be 0.51[31], 0.47[32] and about 0.44 (Duysens, unpublished observation). Clayton[33] provided direct evidence indicating that the absorption peak at 890 mμ was almost completely bleached upon oxidation. Arnold and Clayton[34] made the important observation that the light induced changes in infra-red absorption occurred even at 1°K in dried extracts of bacteria.

In purple bacteria, the bulk of the pigments consists of carotenoids and three bacteriochlorophyll types, called B 800, B 850 and B 890 after the approximate location of the absorption maxima, which vary somewhat from species to species. Only B 890 shows fluorescence. Light energy absorbed by the carotenoids and the other bacteriochlorophyll types becomes only active in photosynthesis through transfer to B 890[4,5]. The fluorescence yield increases upon increasing the intensity of the actinic light. The increase occurs at lower intensities in the absence of hydrogen donors[35]. A quantitative correlation was found under various conditions between the increase in fluorescence and the decrease in absorption around 890 mμ[36]. These observations can be quantitatively explained by the hypothesis that excitation energy is transferred from B 890 to

PH 890; upon receiving a quantum, PH 890 is bleached, yielding P 890. Since energy transfer by induced resonance requires absorption of the energy receiving molecule in the region of the fluorescence spectrum of the energy transferring molecule, the transfer of energy from B 890 to PH 890 stops and the fluorescence yield of B 890 increases. For a simple energy transfer model, if it is assumed that PH 890 does not fluoresce, a linear relation can be derived between the inverse of the fluorescence yield and the increase in absorption. Experimentally, a linear relationship is observed indeed, which supports the hypothesis.[36] This confirms the earlier suggestion of Wassink and coworkers[37] that bacteriochlorophyll fluorescence increases because the "energy acceptor" of photosynthesis becomes exhausted.

In algae and chloroplasts, Kok[38] observed a decrease in absorption at 700 mμ, which was in several aspects similar to the bleaching at 890 mμ in purple bacteria. The bleaching could also be brought about by mixtures of potassium ferri- and ferrocyanide, the E'_o value being about 0.44[39,40]. Kok[41] also observed that the bleaching was brought about most strongly by light absorbed by system 1, and concluded from indirect evidence that the bleaching was reversed by light absorbed by system 2, in a similar way as the oxidation-reduction shifts in cytochrome C 420. Vredenberg (unpublished observations) confirmed this conclusion directly. Thus P 700 is probably located between systems 2 and 1. The high E'_o value and other evidence indicates that P 700 is located between C 420 and system 1. Presumably P 700 is, as Kok suggested, the primary oxidant of system 1. Because of technical difficulties, it was so far not possible in our laboratory to establish a quantitative correlation between the bleaching of PH 700 and the increase in fluorescence of chlorophyll $\underline{a}_1$, i.e. the chlorophyll a of system 1. The main difficulty was, that the fluorescence of chlorophyll $\underline{a}_1$ is small compared to the fluorescence of the chlorophyll a of system 2. There is, however, indirect evidence that chlorophyll $\underline{a}_1$ shows fluorescence and that changes in this fluorescence occur[42,3]; in addition to changes in the fluorescence yield of chlorophyll $\underline{a}_1$ and $\underline{a}_2$, there are also fluorescence changes at 720 and in some species at 730 mμ. The infrared fluorescence is primarily excited by blue light[2,3,5,42] (see also [43, 44]).

Changes in the fluorescence yield of chlorophyll $\underline{a}_2$ and their correlation with a primary photoreductant of system 2.

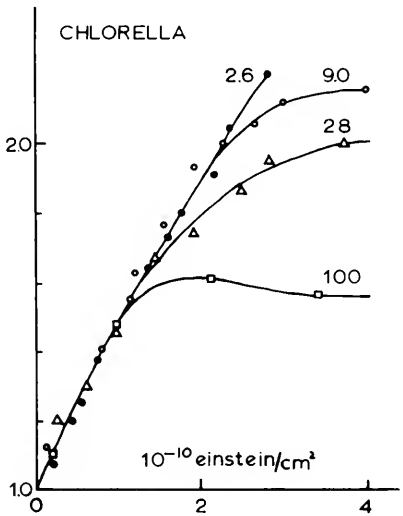
The action spectrum for the excitation of the chlorophyll a

fluorescence in Porphyridium cruentum shows much more pronounced activity of phycoerythrin than of chlorophyll a[4,5], (see also[46]). From this it follows not only that excitation energy is transferred from phycoerythrin to the fluorescing chlorophyll a, but also that the fluorescence is mainly caused by the chlorophyll a of system 2. From the observation that the ratio of the value at the phycoerythrin maximum and that at the chlorophyll a maximum was larger for the action spectrum of chlorophyll a fluorescence than for the absorption spectrum, it had been already earlier concluded that two forms of chlorophyll a occurred. One of these is a fluorescent form, receives its energy to a large extent from phycoerythrin and the other is non- or weakly fluorescent form of chlorophyll a[4,5]. These forms were recently called chlorophyll a_2 and chlorophyll a_1[2].

Upon illumination of a suspension of Porphyridium cruentum, which previously had been in the dark, with green light (light 2) the fluorescence yield rapidly increased, by a factor of about two, and then slowly declined to a steady state value, which was only slightly higher than the value in the dark. The excitation spectrum for this fluorescence increase revealed that it was caused by chlorophyll a_2. If, at the maximum of the increase in fluorescence yield, or shortly thereafter a strong blue beam was admitted in addition to the green actinic beam, the fluorescence yield rapidly dropped to a value not much higher than the steady state value. This phenomenon was observed for all species of algae of different groups investigated: for various red and blue-green algae, the green alga Chlorella and for spinach chloroplasts[3]. Light 2 was found to strongly increase the chlorophyll a_2 fluorescence and light 1 to lower this enhanced fluorescence. If these changes are attributed to chemical changes in a compound Q present in the neighbourhood of chlorophyll a_2, then it can be concluded from scheme 1 that Q is located between systems 2 and 1. Apparently the oxidized form Q quenches the fluorescence, and the reduced form QH does not quench the chlorophyll a_2 fluorescence. DCMU does not inhibit the increase in fluorescence, but it abolishes the decrease in fluorescence (or the reoxidation of QH) by light 1. Apparently Q is different from C 420, P 700, or quinone, since the reduction of these compounds and not the oxidation is inhibited by DCMU. Furthermore the first two compounds are in the dark in general present in the reduced form, while Q is present in the oxidized form. In spinach chloroplasts the chlorophyll fluorescence is strongly enhanced by the addition of a small amount of dithionite. These experiments show that Q is located between

system 2 and the other compounds mentioned, and suggest that QH is the primary photoreductant of system 2.

It was observed (Duysens and Kamp, unpublished experiments) by means of an oscillographic technique that by illuminating a suspension of Chlorella by means of an electronic flash the yield of the fluorescence at 685 mμ mainly due to chlorophyll a_2 could be tripled.



Fluorescence yield ∅ at 685 mμ as a function of total incident energy , for flashes of relative intensities varying from 2.6 to 100. The initial increase depends upon the total energy but not upon the intensity of the flash. For intense flashes for which the risetime of the fluorescence yield is of the order of 10 μsec the maximum level attained decreases.

Fig. 4 shows that the initial increase in a rather wide range of intensities studied, is proportional to the absorbed energy, and independent of the intensity of the flash. If the intensity is sufficiently high that the risetime becomes shorter than 10 μsec, then the yield does not increase up to the maximum attained at lower flash intensities. The shape of the time course depends on the conditions of the algae, but the appro-

ximate proportionality of the initial rate of increase with intensity and the progressive depression of the "steady state" at increasing flash intensities are general phenomena. Apparently this depression is caused either directly or indirectly by a light reaction. In some experiments the fluorescence yield goes through a maximum and decreases appreciably below the value achieved within 10 μsec after the starting of the flash: the strongest depression appeared to occur after about 100 μsec. Whether the depression is caused by a rapid reoxidation of QH by P 700, by another component of system 1, by a short circuit of Z to QH, or by another reaction is a subject of further experimentation.

Photosynthetic phosphorylation

We do not have experimental evidence on the sites of phosphorylation. As far as I know most experimental evidence from other laboratories is consistent with the assumption that noncyclic photophosphorylation(46) occurs between QH and P, and analogy with the respiratory system suggests that plastoquinone and cytochrome may participate in the phosphorylating reactions. The possible sites of phosphorylation are given in the scheme by the arrow with ADP. The fact that cyclic phosphorylation in chloroplasts is, under certain conditions, not inhibited by DCMU suggests then it occurs in dark reactions between XH and P 700. The free energy loss in this redox reaction would be sufficient to permit the production of 2 molecules of ATP per 2 electrons transported, but the measured quantum requirements indicate a two times lower efficiency(47).

SUMMARY

A discussion is given of experimental evidence obtained in our laboratory concerning the mechanism of hydrogen or electron transport in photosynthesis. A scheme is given in which two different photochemical reactions operate in series. Unpublished experiments are presented concerning the presumed primary photoreductant of the system 2 (which system is closely connected to the production of oxygen), and concerning the photooxidation of cytochromes in purple bacteria at low temperatures. It is shown that the initial rate of these photooxidations is decreased upon lowering the temperature, and it is argued that the cytochrome oxidation is a temperature dependent dark reaction. However, at "normal" intensities the rate of this reaction

becomes only limiting at very low temperatures, for some cytochromes at -100°C or even lower.

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CORRELATION BETWEEN ABSORPTION CHANGES AND ELECTRON TRANSPORT IN PHOTOSYNTHESIS

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With the method of sensitive flash photometry we separated and analyzed as yet 7 different types of absorption changes. By this method it was possible to derive a rather detailed reaction scheme on the electron transport system in photosynthesis (1). In the following we will discuss some more details which support the probability of this scheme.

I. PHOTOOXIDATION OF CHLOROPHYLL - a_I

Under "normal" conditions (exciting photosynthesis with red light between 650 - 700 mu) mixed absorption changes can be observed between 400 - 800 mu in chloroplasts and chlorella (s. fig.1 top) (this difference spectrum does not include the absorption changes with life times $< 10^{-3}$ sec).

The changes at 703 mu were discovered by Kok (2). The compound causing these changes he called P 700. He reported also changes at 430 mu in inactivated aceton-extracted chloroplasts (3) and suggested that both changes may be caused by a chlorophyll-a. But according to our measurements (1) there exist at least 5 different types of absorption changes around 430 mu caused by different compounds. Therefore, it is not established that P 700 is a chlorophyll-a. We tried therefore to separate out of the overall difference spectrum in photoactive preparations the spectrum which belongs to P 700 only.

1. Separation of the difference spectrum of chlorophyll-a_I in 5 ways

a) Aged chloroplasts. Aged chloroplasts (spinach) reactivated by addition of reduced TMS (ascorbic acid in excess) show a simplified difference spectrum (4) very similar to that shown in fig.1, bottom.

b) Plastoquinone extracted chloroplasts. Plastoquinone extracted chloroplasts (by petroleum ether) show a simplified spectrum (s.fig.1, bottom), which is very similar to that mentioned under a).

c) Addition of CMU to chloroplasts. CMU-poisoned chlorella or chloroplasts show a difference spectrum which is similar with that of a) and b).

d) Trapping at $-150^{\circ}C$. The difference spectrum of trapped products (4) at $-150^{\circ}C$ of fresh chloroplasts is shown in the

top of fig.2. It shows changes around 703 mu (dotted line) and in addition oxidized cytochrome (solid line). The cytochrome is masking changes around 430 mu which may be caused by other substances. Addition of reduced PMS (phenazine methosulfate) (ascorbic acid in excess), prevents the trapping of oxidized cytochrome (bottom in fig.2). Under these conditions a difference spectrum can be seen at -150° which is similar to that of a), b), and c).

e) A fifth method is described in 7,b).

2. Kinetics

During the flash the absorption decreases very fast ($< 10^{-5}$ sec in chlorella). In the dark a backreaction takes place in $\sim 10^{-2}$ sec at 20°C (fig.5a, 6a) (4)(5).

3. Identification of Chl-a_I

The upper results (5 equal spectra under different conditions) suggest that the changes at 430 mu and 703 mu are caused by one substance. This was additionally proved by comparing the kinetik behaviour of both changes in reactivated aged chloroplasts under different conditions. The lifetime and the magnitude are identical at both wave-lengths at different values of pH (4) (fig.3) and also at different concentrations of added reduced PMS (4). Decreases of absorption changes just with the two absorption bands of chlorophyll-a indicate that very probably a chlorophyll -a (Chl-a_I) is in action (4).

4. Oxidation of Chl-a_I

That the decreases of absorption indicate an oxidation of Chl-a_I in the light, was provided in our experiments by the fact that in aged chloroplasts reduced PMS or reduced DPIP (2,6-Dichlorphenol-indophenol) can be directly coupled to the light product (4)(1). This is demonstrated by the strong acceleration of the decay time with increasing concentrations of red PMS or red DPIP (fig.4) and by the demonstration of a first order reaction (fig.4). The electron-acceptor of Chl-a_I is called Z. Obviously in aged chloroplasts photooxidized Chl-a_I is directly reduced by electrons provided from red PMS:

$$Z \xleftarrow{h\nu} \text{Chla}_I \longleftarrow \text{red PMS}$$

(The arrows indicate the flow of electrons).

5. Chl-a_I - oxidation as a primary act

The production of Chl-a_I^+ within $< 10^{-5}$ sec and the trapping at -150°C give evidence that this oxidation is a primary act (4).

6. The effect of far red background light

Far red actinic light between 700 and 720 mu can only oxidize Chl-a_I (2)(in chlorella and fresh chloroplasts with Hill-oxidants). Actinic light with wave-length $<$ 700 mu results in an oxidation of Chl-a_I followed by a reduction of Chl-a_I^+. Therefore light of $<$ 700 mu is obviously channeled into two reaction

centres. One part - hv_I - is channeled to Chl-a_I (as far red light), a second part - hv_{II} - is obviously channeled to a second reaction centre where it provides electrons from a natural electron-donor for the reduction of Chl-a_I^+ .

$$Z \xleftarrow{hv_I} \text{Chl-}a_I \xleftarrow{hv_{II}} \cdots ?$$

With hv_I-light alone (far red light 7oo - 72o mu) Chl-a_I should accumulate in its oxidized form. Measuring absorption changes at 7o3 mu the measuring beam should already oxidize Chl-a_I. The magnitude of this oxidation must depend on the intensity of the measuring beam (fig.5).

a) In soft measuring light (7o3 mu) practically no Chl-a_I - oxidation takes place. A supplementary red flash ($\lesssim$ 7oo mu) at t_1 with hv_I- and hv_{II}-light has the following effect: The hv_I-light oxidizes Chl-a_I immediately. The hv_{II}-light provides electrons from a second reaction centre for the reduction of Chl-a_I^+. The reduction takes place in $\sim 1o^{-2}$ sec (fig.5a).

b) In medium measuring light (7o3 mu) started at t_2 Chl-a_I accumulates partially in the oxidized form (fig.5b). A supplementary red flash ($\lesssim$ 7oo mu) at t_2 oxidizes the rest of Chl-a_I immediately. Afterwards all Chl-a_I^+ is again reduced in $\sim 1o^{-2}$ sec (fig.5b and 5b'). Therefore, the changes pass the zero line.

c) In stronger measuring light (7o3 mu) started at t_2 nearly all Chl-a_I accumulates in the oxidized form (fig.5c). Therefore in stronger measuring light practically only positive absorption changes take place. These effects are the basic for the following chapter.

7. The electron-acceptor Z of Chl-a_I

Changing from soft to stronger measuring light we should aspect - according to the scheme in fig.5 - a shift from negative absorption changes to positive ones as indicated by fig.5a,b,c. Such a shift has been observed in chlorella (fig.5a and 5b). However, in chloroplasts without any Hill-oxidants in stronger measuring light no shift to positive absorption changes takes place (fig.6 top). This indicates that no accumulation of Chl-a_I^+ has occurred. The reason may be that Chl-a_I^+ can be reduced by a backflow of electrons from the electron acceptor Z:

$$Z \xleftarrow{hv_I} \text{Chl-}a_I \xleftarrow{hv_{II}} \cdots ?$$

If this is true, trapping of electrons of Z should prevent the backflow and allow an accumulation of Chl-a_I^+ :

$$\text{ox.}S_I \xleftarrow{} Z \xleftarrow{hv_I} \text{Chl-}a_I \xleftarrow{hv_{II}} \cdots ?$$

Indeed with the addition of a number of oxidized substances (ox.S_I) positive absorption changes occur which are characteristic for accumulation of Chl-a_I^+ (fig.6 bottom).

This effect gives two important informations.

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a) The shift from negative to positive values indicates which added oxidized substances are electron-acceptors of Z^- (6). So far as investigated all substances surnamed under ox.S_I in fig. 1o react with Z. S.B. Henriques in our laboratory showed that also TPN-reductase - but not TPN alone - traps electrons from Z^-. From the highest redox potential of ox.S_I (methyl viologen) follows for the redox potential of Z^-/Z a value of $\lesssim$ -o,44 Volt (4). Because TPN-reductase is reduced by Z^-, TPN-reductase must contain a redox system (1). Arnon (7) et al have shown that this is ferredoxin. TPN-reductase was already described in detail (7).

b) By shifting the absorption changes of Chl-a_I from negative to positive values* it is possible to separate the difference spectrum of Chl-a_I from the overall difference spectrum (fig.1 top) under complete natural conditions (fig.7) (compare 1,c).

8. Water as the ultimate electron donor of Chl-a_I

It is long known that CMU blocks especially the oxidation of water (9). If water is the ultimate electron donor for Chl-a_I, any reaction of Chl-a_I should vanish in the presence of CMU.

$$ox S_I \longleftarrow Z \xleftarrow{h\nu_I} \text{Chl-}a_I \xleftarrow{h\nu_{II}} \cdots\cdots H_2O$$

This is true for chlorella but this is not the case in fresh chloroplasts (fig.8 mid). The reason is again the backflow of electrons from Z^- to Chl-a_I^+, which keeps the cycle in action. Trapping the electrons of Z^- by addition of oxidized substances ox.S_I (i.e. benzyl viologen), results in the disappearance of the changes caused by Chl-a_I (fig.8 bottom). This fact is a further possibility for the determination of those substances which can accept electrons from Z^- (6).

In aged chloroplasts, where Chl-a_I is supplied directly by electrons of red PMS (see 4), the addition of CMU has no influence on the absorption changes of Chl-a_I (4).

9. Some properties of the Chl-a_I-reaction

a) Redox potential of Chl-a_I. By addition of different ratios of ferro/ferricyanide the ratio of Chl-a_I/Chl-a_I^+ can be changed in the dark. This is shown by the dependence of the magnitude of the light-induced changes at 7o3 mu on the ratio of ferro/ferricyanide. From such measurements Kok (2) estimated a value of + o,43 Volt (pH 6 - 9,5). Our measurements gave nearly the same potential + o,46 Volt (pH 6 - 8) (fig.9) (4). The pH-independence indicates a pure electron transfer.

b) Temperature of inactivation. The Chl-a_I-reaction is stable up to 65°C (1).

c) pH-Range. The Chl-a_I reaction is stable between pH 4 and pH 11 (1).

*) by means of $h\nu_I$-background light (7oo - 72o mu)

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1o. Reaction scheme

The results of this chapter are summarized in fig. 1o (left). In chlorella and fresh chloroplasts Chl-a_I is reduced by electrons originating from water with the help of $h\nu_{II}$-light. After suppressing water-oxidation (by aging, extraction of plastoquinone, addition of CMU), Chl-a_I^+ can be reduced by backflow of electrons from Z^- or by added reduced PMS or reduced DPIP. In these circumstances one light reaction cycle (I) of the overall electron transport system of photosynthesis has been completely isolated (1o)(11)(1). This reaction cycle must be responsible for the system that operated when Vernon, Zaugg and Kamen (12) used aged chloroplasts with reduced substances which are capable of reducing TPN in the light.

II. PHOTOREDUCTION OF X AND PLASTOQUINONE RESPECTIVELY

1. Analysis of X

Out of the overall difference spectrum (under "normal" conditions) we isolated one part: the Chl-a_I-spectrum with peaks at 43o and 7o3 mu.

The other part with peaks around 475 mu and 515 mu has been investigated already in detail (13)(1o). The results are summarized in fig.1o (right). Instead of X we introduced already Q in fig.1o (see below). Part of the changes are caused by a photoreduction of a substance X (redox potential $\sim$ 0 Volt). The electron donor is Y (redox potential $>$ +o,8 Volt). Y oxidizes water. The natural electron acceptor must be according to the result of the last chapter - finally Chl-a_I^+ . Added oxidized substances as surnamed under oS_{II} act as artificial electron acceptors. That the cycle is sensitized by a chlorophyll-a (Chl-a_{II}) follows from the action spectra (11). In the following we will give further evidence for the probability of this scheme.

2. Separation of the difference spectrum of X

To separate the reaction cycle of X from the natural electron acceptor (Chl-a_I), one has firstly to trap the electrons of X by addition of ox.S_{II} (for instance ox.DPIP). To suppress any reaction of Chl-a_I (backflow of electrons from Z^- to Chl-a_I^+), one has secondly to oxidize permanently Chl-a_I by further addition of ferricyanide. Ferricyanide keeps also DPIP permanently oxidized. Fig.11 shows that with addition of DPIP ($1o^{-4}$ M/l) and ferricyanide ($1o^{-3}$ M/l) the difference spectrum of Chl-a_I vanishes and that of X is separated (1o)(2o)(1). In these circumstances a second light reaction cycle (II) of the overall electron-transport system has been completely isolated. This reaction cycle must be responsible for the system which was described by Losada et al (11) (oxygen-producing chloroplasts with ferricyanide and DPIP).

3. Relation of X and Plastoquinone-content

a) Bishop (14) has shown that plastoquinone is somewhere involved in the electron transport system of photosynthesis. The

redox potential of plastoquinone ($\sim$ 0 Volt) is very similar to that of X ($\sim$ 0 Volt). This encouraged us to look for the relation of X and plastoquinone.

b) Petroleum ether extraction of chloroplasts (fig.12) results in a decrease of the absorption changes of X. Following condensation of synthetic plastoquinone results in a complete reappearance of the difference spectrum or even more (15).

Both results indicate that X is closely related or identical with plastoquinone (15). But plastoquinone in vitro is not accompanied by absorption changes at 475 and 515 mu. If X is identical with plastoquinone, we have to assume that in vivo the reaction of plastoquinone influences the absorption of its surrounding pigments, resulting in changes at 475 and 515 mu.

4. The difference spectrum of plastoquinone

Absorption changes which are directly related to the reduction of pure plastoquinone in vitro show changes with a maximum at 254 mu (16). Corresponding changes should occur in vivo. We have observed light induced changes in active chloroplasts (17) which correspond to those in vitro.

5. Separation of the difference spectrum of plastoquinone

We reported that with addition of DPIP (ferricyanide in excess), the difference spectrum of Chl-a_I vanishes while that of X is furthermore in action. Because of the demonstrated relation of X and plastoquinone we supposed that under these conditions also the difference spectrum of plastoquinone should be still in action. Measurements at 254 mu show indeed that with addition of DPIP and ferricyanide the magnitude of the changes at 254 mu are influenced not at all while those of Chl-a_I at 703 mu are completely quenched.

6. Relation between absorption changes of X and plastoquinone

The absorption changes of plastoquinone (254 mu) and X (475 and 515 mu) were measured in chlorella and chloroplasts (with DPIP + Ferricyanide) in relation to light intensities, duration of the flash, temperature and pH. We added also different concentrations of CMU and changed the content of plastoquinone by different extractions. Under all these conditions the magnitude as well as the time course at 254 mu and 515 mu correspond to each other. In fig.13 the changes at 515 and 254 mu are compared as function of extraction and recondensation of plastoquinone. Fig.14 shows the decrease of both changes with increasing extractions of plastoquinone. The changes of Chl-a_I (upper curve) at 703 mu are influenced not at all. This indicates in a different way (compare 5) that plastoquinone seems not to be involved in the reaction cycle of Chl-a_I.

7. X and plastoquinone respectively as electron acceptor of water oxidation

That X is the electron acceptor of lastly water in reaction cycle II, we gave in the last years several evidences. A further

proof that X and plastoquinone resp. is the electron acceptor of water is given in the following experiment: We measured simultaneously with the extraction and recondensation of plastoquinone and the corresponding disappearance and reappearance of the absorption changes at 515 mu the oxidation of water (by O_2-production measurements). Comparing such results (fig.15) it can be seen that the magnitude of the changes at 515 mu parallels the rate of O_2-production. On the other hand the changes at 515 parallels the changes at 254 mu (fig.13, 14).

8. Reaction scheme

The results show that plastoquinone reacts at the position of X. X is identical with plastoquinone or closely related to it. Therefore we introduce in the already shown scheme instead of X plastoquinone Q (18)(15).

III. THE ACTION SPECTRA OF THE TWO SEPARATED REACTION CYCLES I AND II

The action of cycle I is optically represented by the absorption changes of Chl-a_I at 430 mu and chemically by the rate of reduction of oxS_I, (i.e. benzylviologen). The action of cycle II is optically representated by the magnitude of the absorption changes at 515 mu and chemically by the rate of oxidation of water (O_2-production).

Fig. 16 (top) show the optically measured action spectra (11) of the two separated cycles and on the bottom the chemical action spectra are shown. (The optical action spectra could be measured with a much higher precision than the chemical ones. The chemical action spectra are preliminary ones.) The results are in agreement with the Emerson-effect (20): The peaks indicate those types of chlorophylls which provide the two reaction centres Chl-a_I and Chl-a_{II} per energy migration with light energy (11). These different types are noted in fig.10. The long wave-length limit for reaction cycle I is at $\sim$730 mu, the long wave-length limit for reaction cycle II is already at $\sim$700 mu. The action spectrum for the reduction of plastoquinone was furthermore investigated directly at 254 mu. It has a long wave-length limit at 700 mu in accordance with the measurements at 515 mu and of oxygen production.

IV. THE COUPLING OF REACTION CYCLE I AND II

1. First demonstration

Optically demonstrations of the existence of two coupled light reactions were done in the following way: Kok (2) showed that far red light causes oxidation of P 700 at 703 mu and shorter wave-length its reduction. Duysens (21) showed in red algae that red light causes oxidation of cytochrome and shorter wave-length its reduction. We showed in green plants (18) that far red light causes oxidation of cytochrome while shorter wave-length causes the reduction of X which reduce cytochrome in the dark.

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The above reported results show that when under natural conditions (chlorella) there exists a coupling between the reaction cycle I and II, it should be possible to demonstrate the coupling of the cycles I and II through the absorption changes of Chl-a_I directly at 430 mu. (Because of the strong fluorescence in the red region a coupling at 703 mu can directly not be demonstrated).

On the other hand we selected with plastoquinone a first chemical representative of cycle II and it should be possible to demonstrate the coupling of cycle I and II also at 254 mu.

According to the action spectra Chl-a_I can be oxidized with light $\geq$ 700 mu, i.e. 720 mu (decrease at 430 mu) and Chl-a_I should be reduced with $\leq$ 700 mu light, i.e. 634 mu (increase at 430 mu). 634 mu light produces namely reduced plastoquinone (Q^-) and when there exists a coupling between reaction cycle I and II, Q^- should provide Chl-a_I^+ with electrons. Vice versa plastoquinone can be reduced with $\leq$ 700 mu light, i.e. 634 mu (decrease at 254 mu) and Q^- should be oxidized with $\geq$ 700 mu light, i.e. 720 mu (increase at 254 mu). 720 mu light produces namely Chl-a_I^+ (see above) which accepts the electron of Q^- when there exists a coupling between reaction I and II. The results in fig. 17 confirm these predictions.

2. Second demonstration

It should be pointed out that as yet in all cases coupling of two light reactions in photosynthesis was demonstrated only by two different light colours. The following experiments show a different and completely independent demonstration of coupling.

We separated chemically the reaction cycle I from cycle II and investigated the different properties of these separated reactions (chloroplasts of spinach) (1o)(2o)(1).

Some typical properties of reaction cycle I are for instance: a) pH-range between 4 - 11; b) independence from the presence of CMU. (CMU blocks water oxidation); c) practically independent from aging.

Corresponding properties of reaction cycle II are: a) pH-range between 5 - 8; b) strong dependence from the presence of CMU; c) strong dependence from aging.

When however both cycles are completely coupled (fresh chloroplasts with Hill-oxidants ox.S_I or whole chlorella cells), the behaviour of reaction cycle I (demonstrated for instance by absorption at 703 mu) and the behaviour of reaction cycle II (demonstrated for instance by absorption changes at 515 mu)should show one and the same dependence as function of pH, CMU age, etc.

Figs.18-2o show the result. The influence is the same at both wave lengths (6). This proves in a completely different way the quantitative cooperation between both reaction cycles (6).

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V. A POOL OF PHOTOACTIVE PLASTOQUINONE

From the magnitude of absorption changes of Chl-a_I at 703 mu and of Q at 254 mu it follows (together with the extinction coefficients of pure chlorophyll and pure plastoquinone) the ratio of Chl-a_I: Q $\approx$ 1 : 4. Q is the photochemical engaged plastoquinone; the total amount of plastoquinone is ten times higher (22). This means that there exists a pool (reservoir) of photoactive plastoquinone between both light reactions (s.fig.21) shuttling electrons from reaction centre I to II. This result is also in accordance with the fact that after extraction of plastoquinone this substance can be recondensed into the same position. This seems to be possible only when there exists a gap between reaction centre I and II which is filled up with larger amounts of plastoquinone. The redox potential of this pool is probably controlled by oxidants (O_2) as well as by reductants. This pool seems to be the reason for most of the so-called "induction phonomena" in photosynthesis.

VI. REACTION MECHANISM OF THE OVER ALL PROCESS

The coupling of reaction cycle I and II leads to a reaction scheme of the over all process of the electron transport in photosynthesis which is mapped out in (1o)(1). An intermediate between both cycles is probably cytochrome. This was demonstrated in (18)(4)(1o)(1).

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Fig. 1. Spectra of flash induced absorption changes in suspensions of normal (top) and petroleum ether extracted chloroplast (bottom) from spinach. pH = 7,2; 20°C.

Fig. 2. Spectra of the light induced irreversible absorption changes in spinach chloroplast fragments at -150°C in the absence (top) and in the presence of red PMS (bottom).

Fig. 3. Half-life of the absorption changes at 435 mu and 705 mu in aged whole spinach chloroplasts as function of pH. 20°C, additions: 10^{-5} M/l PMS and 3.10^{-3} M/l ascorbate.

Fig. 4. Log. plot of the relative absorption changes at 705 mu in aged whole spinach chloroplasts vs. time at different PMS concentrations ($+3.10^{-3}$ M/l ascorbate in excess). pH = 8, 20°C.

Fig. 5. Bottom: Time course of the absorption changes at 705 mu in chlorella cells induced by short flash ($\sim 10^{-3}$ sec) of red light at t = 0. a' in soft and b' in medium measuring light. 20°C, pH = 7,8. For further details see text.

Fig. 6. Time course of the absorption changes at 705 mu in whole spinach chloroplasts without additions (top) and with added benzylviologene as e-acceptor (bottom). 20°C, pH = 7,2. The light flash ($\sim 10^{-5}$ sec) was turned on at t = 0.

Fig. 7. Time course of the absorption changes at 705 mu and 430 mu in whole spinach chloroplasts in the presence of 10^{-4} M/l benzylviologene with soft measuring light and continuous 710 mu background light. 20°C, pH = 7,2. Both absorption changes were induced by a flash ($\sim 10^{-5}$ sec) at t = 0.

Fig. 8. Time course of the absorption changes at 705 mu in whole spinach chloroplasts in the absence and in the presence of CMU and CMU plus benzylviologene. 20°C, pH = 7,2. The flash ($\sim 10^{-5}$ sec) was turned on at t = 0.

Fig. 9. Redox titration of the photo-induced absorption changes at 705 mu in aged spinach chloroplast fragments. Log. plot of $(\Delta I_{max} - \Delta I)/\Delta I$ vs. the redox potential of added ferri-/ferrocyanide mixtures at different pH-values of the suspension. Ferri- + ferrocyanide concentration $\approx 10^{-2}$ M/l, 20°C.

Fig. 10. Scheme of reaction cyclus I (left) and of reaction cyclus II (right). For details see text.

Fig. 11. Spectra of the flash-induced absorption changes in spinach chloroplast fragments in the absence (top) and in the presence (bottom) of 10^{-4} M/l DPIP plus 10^{-3} M/l ferricyanide. 20°C, pH = 7,2.

Fig. 12. Spectra of the absorption changes in spinach chloroplast fragments under normal condition, after extraction with petroleum ether and after recondensation of plastoquinone. (1) soft, (2) medium, and (3) strong extraction. 20°C, pH = 7,2.

Fig. 13. Time course of the absorption changes at 515 mu and 254 mu in spinach chloroplast fragments under normal condition (top), after partial extraction with petroleum ether (middle) and after recondensation of plastoquinone (bottom). 20°C, pH = 7,2. The flash ($\sim 2.10^{-2}$ sec) was turned on at t = 0.

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Fig.14. Absorption changes at 705 mu, 254 mu and 515 mu in spinach chloroplast fragments as function of different degrees of extraction with petroleum ether. 20°C, pH = 7,2.

Fig.15. Rate of O_2-production by illuminated spinach chloroplast fragments and magnitude of the absorption changes at 515 mu in the same preparation of chloroplast fragments. Normal condition, after extractions with petroleum ether different degrees, and following recondensation of plastoquinone. 13°C; pH = 7,2; ferricyanide was added as Hill oxidant.

Fig.16. Action spectra of reaction cycles I and II. Top: Measured by the magnitude of the absorption changes at 433 mu in aged spinach chloroplast fragments in the presence of 10^{-4} M/l PMS plus 10^{-2} M/l ascorbate (20°C, pH = 7,2) and at 515 mu in normal spinach chloroplast fragments in the presence of $1{,}5.10^{-5}$ M/l DPIP plus 10^{-2} M/l ferricyanide (20°C, pH = 7,2). Bottom: Measured by the rate of O_2-production of illuminated spinach chloroplast fragments in the presence of 2.10^{-5} M/l DPIP plus 6.10^{-3} M/l ferricyanide (20°C, pH = 7,2) and measured by the rate of O_2-consumption of illuminated aged spinach chloroplast fragments in the presence of 10^{-4} M/l DPIP plus 5.10^{-3} M/l ascorbate plus 3.10^{-5} M/l benzylviologene (20°C, pH = 8). (Measured by U.Siggel in our laboratory).

Fig.17. Top: Time course of the absorption changes at 430 mu in spinach chloroplast fragments in the presence of 10^{-4} M/l benzylviologene illuminated with 720 mu-light (1/2 sec), followed by 638 mu-light (10^{-4} sec) (20°C, pH = 7,2). Bottom: Time course of the absorption changes at 254 mu in chlorella cells illuminated with 638 mu-light (1/5 sec), followed by 720 mu-light (1 sec) (20°C, pH = 7,2).

Fig.18. Top: Absorption changes at 515 mu and 705 mu in whole spinach chloroplasts in the presence of 10^{-4} M/l ferricyanide as function of aging. (20°C, pH = 7,2). Bottom: Absorption changes at 515 mu and 705 mu in whole spinach chloroplasts in the presence of 10^{-4} M/l ferricyanide as function of pH (20°C).

Fig.19. Half-life of the absorption changes at 515 mu and 705 mu (strong measuring light) in chlorella cells in dependency of temperature (pH = 8). The chlorella culture differs from that used in the measurements of fig.5. (In chloroplasts identical kinetics at 515 mu and 705 mu could as yet not be established).

Fig.20. Absorption changes at 515 mu and 705 mu in whole spinach chloroplasts in the presence of 10^{-6} M/l indigocarmine as function of concentration of added CMU. (20°C, pH = 7,2).

Fig.21. See text.

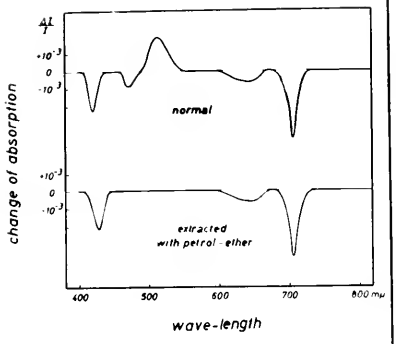


fig. 1

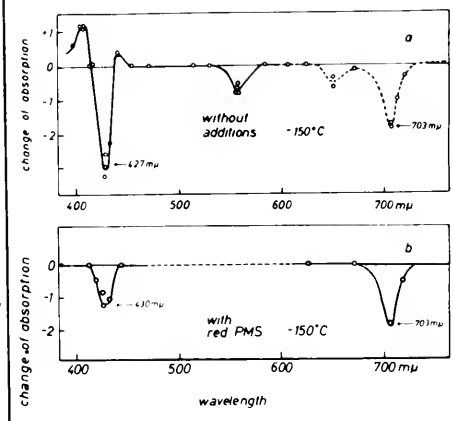


fig. 2

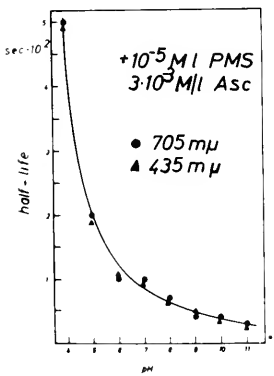


fig. 3

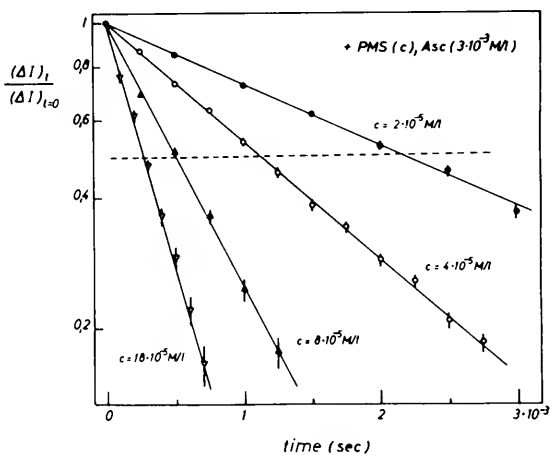


fig. 4

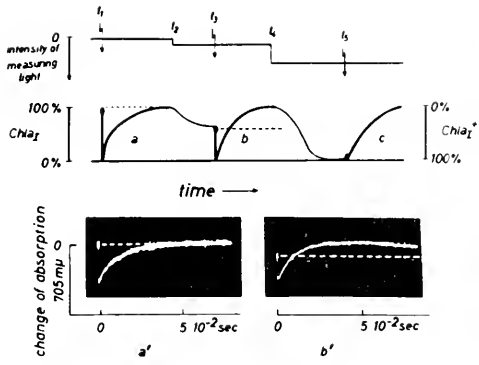


fig.5

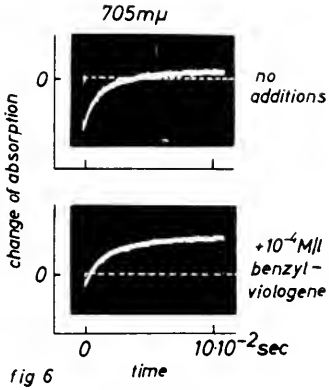


fig 6

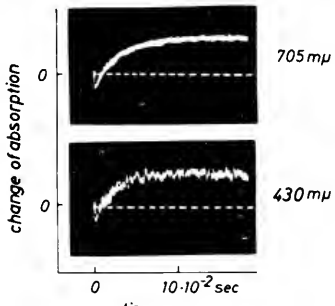
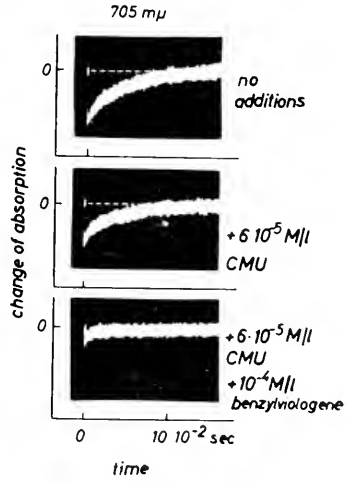


fig. 7



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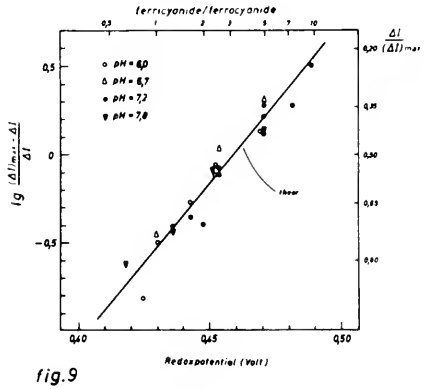
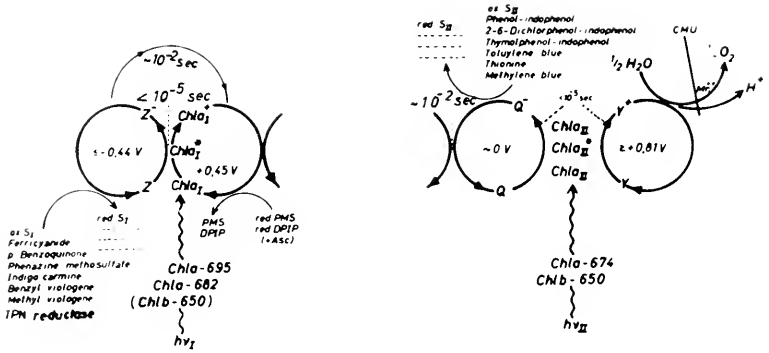


fig.9



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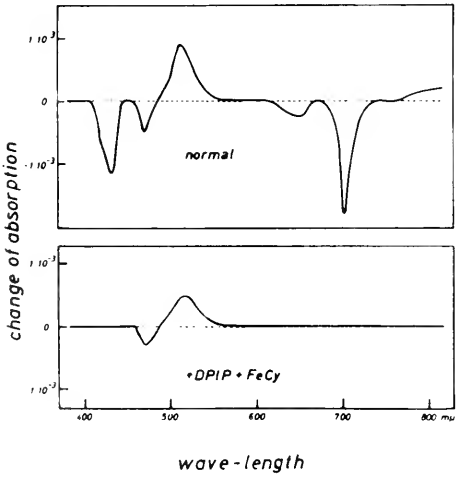

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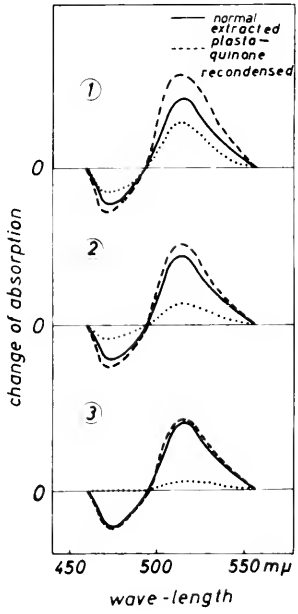
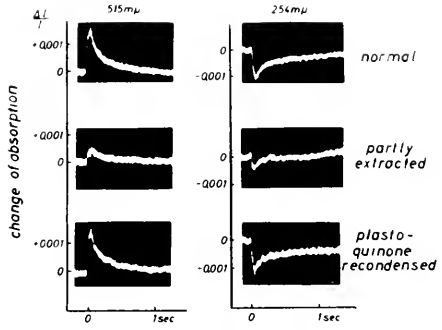


fig.12



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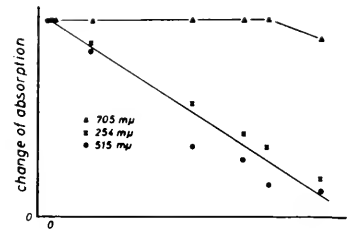

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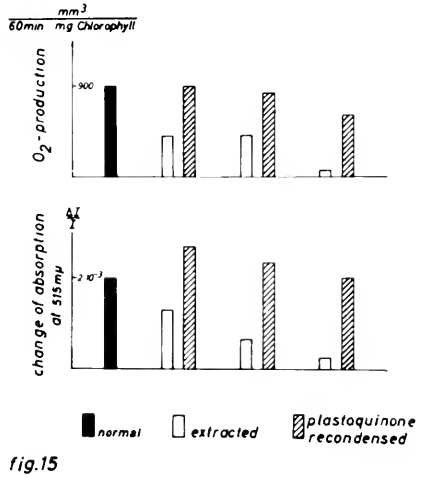

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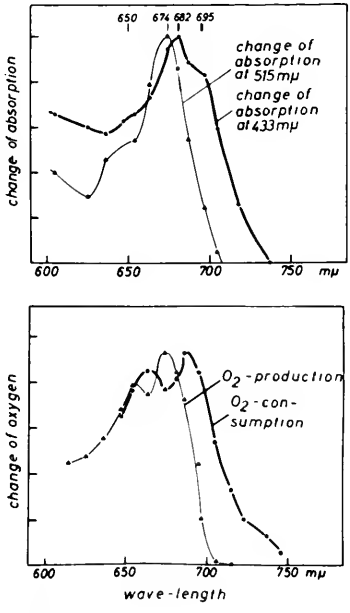

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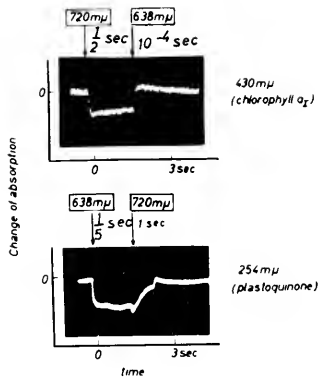

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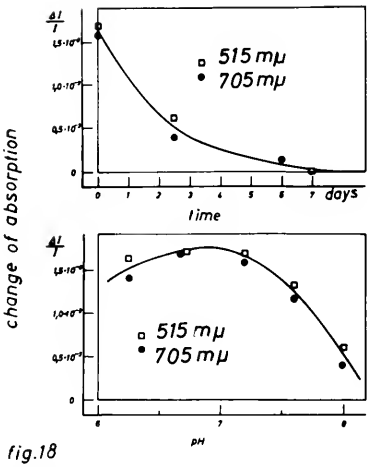

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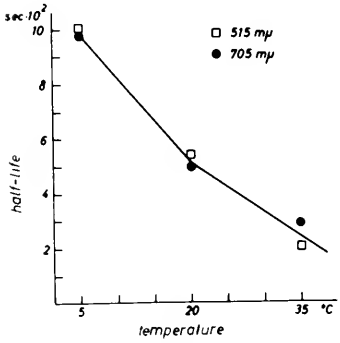

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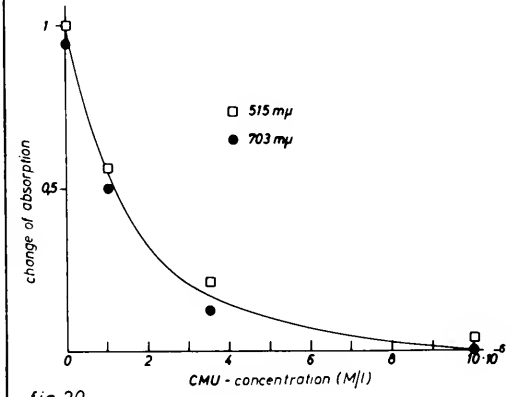

fig.20

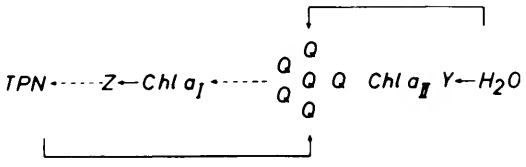

fig.21

PHOTOSYNTHETIC ELECTRON TRANSPORT

Bessel Kok

I. The first photoreaction of photosynthesis mediated by P700

It is well established that in aerobic photosynthesis two photoreactions occur. Photosystem I is sensitized by "long wave" chlorophyll which feeds absorbed quanta into a special long wave pigment "P700" (13). Upon excitation P700 loses an electron and remains in a bleached form which exerts the properties of a weak oxidant (normal potential $P/P^{+} = 0.43$ volt). The electron acceptor ("X^{-}") is a strong reductant of a potential lower than -0.42 volt (one can show that it reduces methylviologen). Figure 1 and Table 1 bring quantitative evidence that photoact I is mediated by P700: Fig. 1 shows an experiment made by Hoch and Martin (1) with fresh chloroplasts. The reduction of TPN was studied as a function of

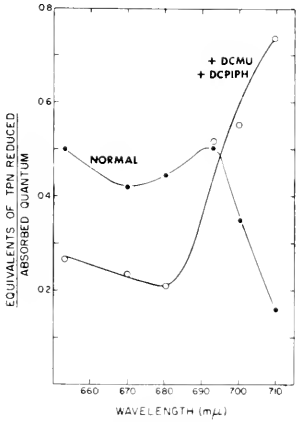

Figure 1

Equivalence Between Rates of P700 Turnover and TPN Reduction

Reaction mixture (pH 7.8) contained per ml (in μ moles):
Chlorophyll : 0.017 ; TPN : 0.5 ; ADP : 1 ; PO_4 : 10 ;
Mg^{++} : 2.5 ; PPNR : saturating
Illumination 710 mμ

$$\mu \text{eq./min } P_{700} = \frac{\Delta \text{O.D. } 700 \text{ (A-B signal)} \times 1800 \text{ (RPM)}}{80\,000 \text{ (E mol.)} \times \text{mg Chlorophyll}}$$

$$\mu \text{eq./min } TPNH_2 = \frac{\Delta \text{O.D. } 340 \text{ (B-W signal)}}{3100 \text{ (E mol/2)} \times 1 \text{(min)} \times \text{mg Chlorophyll}}$$

P 700	TPN	Ratio
2.1	2.1	1

Table 1

wavelength both in the absence and the presence of the poison DCMU. Reduced indophenol was added in the latter case to relieve the DCMU inhibition (2). In the unpoisoned chloroplasts, two quanta of 650-680 mu light, are required to transfer one electron to TPN, the quantum yield drops severly in wavelengths beyond 690 mu. In the presence of DCMU and reduced dye, however, the quantum yield is relatively low at

short wavelengths but rises with increasing wavelength until it approaches unity (1 eq./hν). Obviously, in long wave light we observe only photosystem I, a process which occurs with a quantum yield of one and can retain a considerable fraction (≥ 50%) of the absorbed light as chemical energy. The experiment described in Table 1 was made with our difference spectro-photometer (3) in which a sample is exposed to a series of light flashes (1800 per minute). The dark periods are long enough to allow dark conversion of the photoproducts made in each flash. The apparatus measures cyclic absorbency changes of intermediates as well as the net result of many cycles, provided a color change accompanies the events. In Table 1 we observed the repetitive bleaching (in each flash) and re-reduction (in the dark) of P700. If for every P700 which is bleached, one electron is transferred to the primary reductant (X) and from there to TPN, then the total amount of $TPNH_2$ accumulated should correspond to the sum of all P700 molecules which have turned over during the time the flashing light was given. This indeed proved to be the case if we assumed a (change of) molar extinction $\Delta E = 8 \times 10^4$ L/mole. cm. for P700 -a value typical for the red absorption band of a chlorophyll. This good agreement, together with the high quantum yield observed in Fig. 1 yields quantitative support to the proposal that P700 is the photoconverter of photosystem I in photosynthesis.

II. Photoreduction and -oxidation of indophenol dye, site of phosphorylation.

In the long wave light or in the presence of DCMU the second photoreaction of photosynthesis is inoperative. Earlier (4) we have shown that DCMU does not really inhibit the photoreduction of DCPIP by chloroplasts, but only causes the reduced dye to react back with photo-oxidized P700 (whose

Reduction of DCPIP by weak flashing light of two wavelengths

The amount reduced per flash is compared to the net rate during one light-dark period.

725 mμ values in parenthesis are corrected for absorbed intensity equal to the 674 light.

Wavelength actinic light mμ	Fractional absorption σ_0	Rate per flash 10^{-5} O.D.	Rate per 1/30 sec 10^{-5} O.D	Ratio flash yield net rate
674	≥ 80	49	33	1.5
725	≈ 6	9 (≈ 100)	0.28(≈ 3)	32

Table 2

normal reduction by the second photoact is impeded by the poison).

Table 2 shows that essentially the same happens in long wave light: In this experiment, performed like the one shown in Table 1, we simultaneously measured the amount of dye reduced in each flash--whether reacting back or remaining in the reduced form--and the net amount of DCPIPH accumulated by all flashes.

If N flashes of 674 mu light are given, an amount of DCPIPH accumulates which nearly equals N times the amount reduced per flash. However, if 725 mu flashes are given instead, only 1/30 of this amount accumulates, 97% of the reduced dye is re-oxidized in the dark periods by $P700^+$. The long wave drop of the quantum yield of dye reduction (5) therefore is due to a lack of the normal reductant generated by photosystem II which reacts with $P700^+$ much faster than DCPIPH. Actually, if one corrects for the low fractional absorption of 725 mu light, the data of Table 2 show that in this wavelength area dye is reduced with twice the quantum yield observed in 674 light (analogous to the reduction of TPN in expt. Fig. 1).

We may conclude that in these discussed experiments DCPIP is reduced at the same locus as TPN: by the primary reductant X^- made in the first photoact. It is clear then that in the absence of the second photoact (DCMU or long wave light), indophenol dye mediates a vigorous cyclic electron transport. Trebst and Eck (6) showed that this cycle is coupled to ATP formation and therefore must include the site of photophosphorylation.

We agree with Witt, et. al. (7) that at least in fresh chloroplasts DCPIPH does not reduce $P700^+$ directly but via another intermediate. It remains to be proven that this intermediate is cytochrome b_6 or plastoquinone such as would be required by hypotheses which correlate one of these components with photophosphorylation. The high normal potential of the dye and the considerable concentration of the oxidized form which can result from its photo-oxidation (even in the presence of ascorbate cf. 4 Table 1) argue against this possibility. A more likely site for reduced dye to re-enter the cycle is cyt. f. From an energetic viewpoint, this would exclude ATP formation at this locus (cf. 4).

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III. Photo-oxidation of cytochromes and plastocyanin by detergent treated chloroplasts.

Photosystem II is quite sensitive to ageing, heating or detergent. Photosystem I, however, survives such treatments to a considerable extent. Possibly by opening up the chloroplast structure, detergent makes $P700^+$ accessible for large molecules such as cytochrome c, f, or plastocyanin so that they can be photo-oxidized (like reduced indophenol dye). The photo-oxidation of cytochrome c by this material was first observed by Niemann and Vennesland (8). We may briefly summarize the results of our own recent studies of such photo-oxidations (9). The process is sensitized quite efficiently by long wave light (the yield approaches 1 eq./$h\nu$) involving photosystem I--$P700^+$ being the photo-oxidant. Two types of catalysts accelerate the photo-oxidation of ferrocytochrome c:

Viologen, flavin and other auto-oxidizable, single electron transfer agents stimulate by mediating between primary reductant X^- and oxygen.

Cytochrome f (Cyt. 552 from Euglena) and plastocyanin (10) stimulate by mediating between $P700^+$ and ferrocyt. c. Figure 2 illustrates the effect of various concentrations of plastocyanin upon the rate of cyt. c photo-oxidation in the presence and absence of viologen. Rates approaching 5000 eqs./chl. hour have been observed, severalfold higher than found in

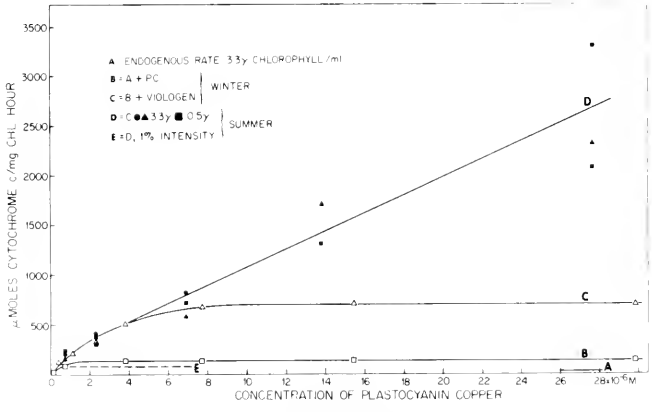

Figure 2

photosynthesis or chloroplast reductions. The rates proved remarkably independent of temperature. ($Q_{10} \simeq 1.3$, + 30°--5°C) Cyt. f acts very similar to plastocyanin but never

yielded rates higher than ~800 eq/chl. hour. A peculiarity of these catalysts is their effectiveness in weak as well as in strong light--they increase the quantum yield of the reaction.

Plastocyanin and cyt. f, if added in substrate amounts instead of cyt. c are photo-oxidized themselves with high rates. An interesting feature of these reactions is that the rate is dependent upon the redox state of the substrate. For instance, if cyt. f is added in half-oxidized, half-reduced form, the rate is only half maximal. (In the presence of excess cyt. c, cyt. f is kept in the reduced form and the overall reaction proceeds with optimal rate until the depletion of cyt. c.)

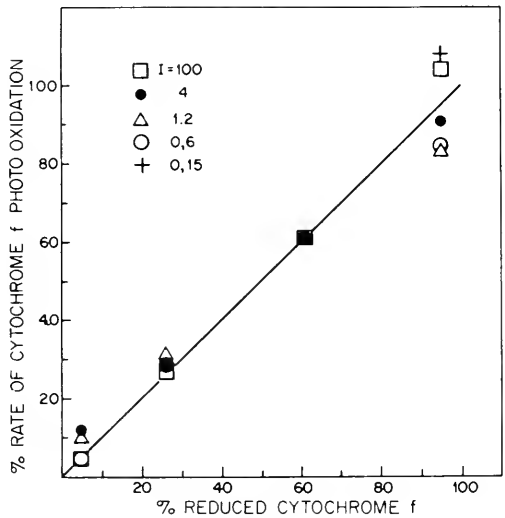

Figure 3

Figure 3 illustrates that in weak as well as in strong light, the rate of cyt. f photo-oxidation is proportional to the ratio cyt. f red./cyt. f total. To explain this, we assume that external cyt. f equilibrates with a cyt. f molecule fixed in the chloroplast matrix in close proximity to P700. A photoact can only be successful if not only P700 but also its associated cyt. f is in the reduced state before the quantum hits. The cytochrome transfers an electron to $P700^+$ immediately after the latter has lost its electron to X, and thus prevents a back reaction between the photoproducts. Such a charge transfer complex between cyt. f and P700 embedded in the chloroplast matrix was already shown by Witt et al. (7). In scheme Fig. 4 we indicated that plastocyanin could react in two ways with $P700^+$, directly or via

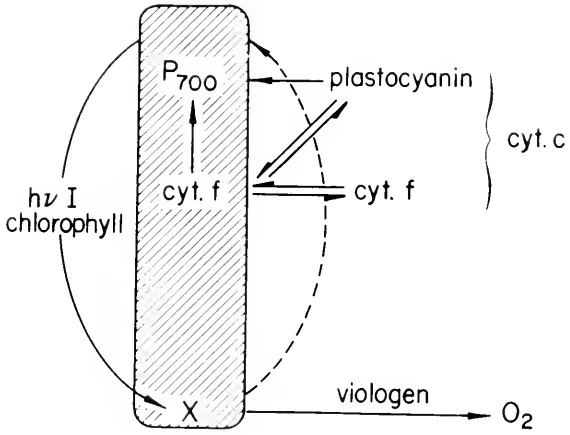

Figure 4

cyt. f. In many chloroplast preparations plastocyanin sustained much higher photo-oxidation rates than cyt. f, indicating, that it bypasses cyt. f and reacts directly with P700. Our preliminary data indicate that the kinetics of plastocyanin photo-oxidation do not essentially differ from those of cyt. f oxidation (cf. Fig. 3). This suggests that not only cyt. f, but also plastocyanin has a fixed locus in the chloroplast matrix and can operate in a complex with P700. This intimate cooperation between the photoreceptor, a haem and a copper enzyme, all of high potential, present in equal amounts in the chloroplasts, has prompted some speculations which will be described in the following section.

IV. Discussion

The first photoact yields besides X^- (re-oxidized by substrate) a weak oxidant $P700^+$. The second (short wave sensitized) photosystem is left with the tasks to evolve oxygen (at least to assist this process) and at the same time re-reduce $P700^+$. One of the products of the second photoact, therefore, must be a reductant "Y^-" of a potential lower than +0.43 volt. Just how much lower, is presently a point of discussion since the chemical nature of Y is unknown. For instance, as mentioned by Dr. Hoch in this symposium, the possibility is not excluded that Y^- is as strong a reductant as X^-. This would mean a "parallel" operation of the two photoacts and require an energetic coupling between them by dismutation reactions in the dark.

The other extreme is the assumption that the potential of

Y/Y^- is only slightly lower than that of P700, i.e. the second photoact is strictly in "series" with the first one. We have mentioned this possibility on an earlier occasion (4) and illustrate it again in scheme Fig. 5.

These two extremes share one feature, namely, that the quantum requirement of the overall process could be less than 2 per electron (<8 per O_2): We feel the data of Fig. 1 and Table 1 definitely show a requirement of one quantum to generate X^- and P^+. In the parallel formulation the extra energy required to evolve O_2 starting from P^+ does not necessarily have to come in one quantum "packages" and in the series formulation the energy of 2 (short wave) quanta might suffice to evolve oxygen from a preformed complex.

A minimum requirement of 2 hv/el. is more rigidly demanded by the part-parallel part-series scheme [cf. Hill and Bendall (11)] which is presently accepted by most workers in the field. In this formulation the reductant of the second photoact

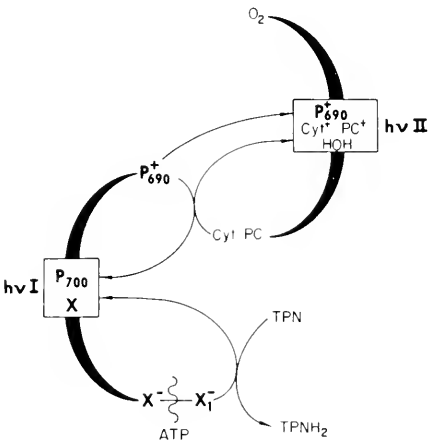

Figure 5

should be able to reduce cytochrome b_6 or plastoquinone (potential of $Y/Y^- \leq 0.0$ volt). The reaction chain connecting the two photoacts:

$$Y^- \longrightarrow \text{P.Q.} \xrightarrow[\text{ADP}]{\text{ATP}} \text{cyt. f} \longrightarrow P^+$$

provides an attractive site for photophosphorylation--coupled to the main stream of electron transport. If one does not

accept this intermediate chain $Y^- \longrightarrow P^+$ (cf. section II) the only reasonable alternative to conceive a generation of ATP coupled to substrate reduction is the locus indicated in scheme Fig. 5 as $X^- \longrightarrow X_1^-$ (4). This hypothesis of "substrate level" photophosphorylation requires, however, that the primary reductant of the first photoact has a quite low potential.

Scheme Fig. 5 contains additional speculations which must appear objectionable to many of you and might not survive future evidence. However, it is not presented as the ultimate truth but rather to illustrate some possibly useful thoughts. One feature is a "sharing" of traps by the two photoreactions visualized by the identification of $P700^+$ with the light collector of photoreaction II, i.e. Duysens' "Q" (12) and Witt's "Q II" (7). The asymmetric shape of the 700 mu difference band (cf. 13, Fig. 2 and 13^b) suggests a shift of the 700 mu absorption to a (~50%) weaker band at a (~13 mu) shorter wavelength. Photo-oxidized P700 ($P690^+$) thus could conceivably again function as a light trap--compare e.g. the photochromic back and forth shift of the plant pigment phytochrome (14). The second photoact then would be sensitized by all wavelengths except those beyond 690 mu (of course, one must assume that P690 undergoes alternate dark steps as well).

Such a competitive sharing of trapping pigment by the two photoacts allows a self-regulating sensitization mechanism ("spill over"), in line with earlier thoughts of Franck (15) and Myers (16). Our fluorescence data, although supporting this spill over from one photosystem to the other, do not seem to favor an identification of the conversion centers and indicate a more complex type of mechanism (this volume).

A main argument for closely connecting the two traps i.e. for a short path between the intermediate products of the two photoacts--regardless of their nature--is the following: The spectroscopic evidence such as obtained by Witt, Duysens, and ourselves for P700 operating in a charge transfer complex with cyt. f and probably plastocyanin, subject to a push-pull operation by two photoacts, is impressive. However, most of the information originates from measurements under extreme or abnormal circumstances. Under favorable conditions of efficient, steady state photosynthesis in whole algae, neither P700 nor cyt. f can be observed by our flashing light method which is geared to the "time constant" of photosynthesis and has a time resolution of a millisecond. With their much

faster method Witt et al. (7) still fail to observe the reduction of cyt. f, presumably carried out by a dark step following the second photoact. (Spectroscopic measurements which use continuous actinic illumination cannot discriminate between compounds directly involved in the electron transport chain and those which are indirectly affected.) Unless one considers a complete re-interpretation of available data, one must assume that the early events in photosynthesis occur with such extreme rapidity that they escape observations with present methods. Also, this feature seems to leave little room for an intermediate electron transport chain $Y^- \longrightarrow P^+$.

Finally, one cannot help drawing an anology between the (copper and haem containing) cytochrome oxidase in respiration and the peculiar combination of plastocyanin and cytochrome f in photosynthesis (cf. section III)--reason why we have suggested in scheme Fig. 5 that photosynthetic oxygen evolution might be a light driven reversal of the terminal respiration step (4).

Presently, the evidence concerning the photosynthetic light reactions is so much in flux and of qualitative nature that the speculations forwarded do not appear too much out of line.

V. Acknowledgements

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FLUORESCENCE STUDIES

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I. A photoinitiated emission at 698 mu.

Brody and Brody (1) mentioned that upon cooling to 77°K the 685 mu fluorescence maximum shifts to 690 mu. We observed, however, that the location and intensity of the 685 mu band ("F685") are practically independent of temperature. Below -150°C a distinctly separate band develops at 696-698 mu (denoted "F700") which keeps increasing with decreasing temperature until in many cases it is the most pronounced emission (cf. Figs. 2, 4, and 5). This emission occurs in all organisms we have investigated, in acetone extracted or detergent treated chloroplasts, but not in solutions of chlorophyll.

In fresh chloroplasts, leaves or algae, a striking feature of this emission band is a requirement of light in order to fully develop. Figure 1 (top curve) shows an experiment made in the following way: a sample of chloroplasts was kept in

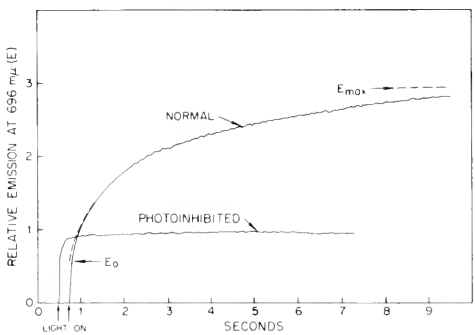

Figure 1

the dark for a few minutes and then cooled to liquid nitrogen temperature--also in darkness. Upon addition of the exciting light, the fluorescence intensity rises instantaneously to a certain level (E_o) and from thereon much more slowly until it reaches a final value (E_{max}).

An immediate rise of fluorescence is typical for pigment in solution, for most of the low temperature emission at 730 mu ("F730") and also for the 685-697 bands in aged or heated chloroplasts. It indicates that already, before light is

given, the responsible pigment molecules are in the fluorescent state. The exponential build-up of part of the 698 emission in fresh leaves or chloroplasts implies that the responsible pigment is not in the fluorescent state to begin with, but converted into it by light. We therefore, must assume the presence of trapping centers which cease to be traps as soon as they have received a quantum.

One can conceive two possible mechanisms: (a) the trapping pigment bleaches upon excitation and ceases to absorb light from surrounding pigment so that the latter is free to fluoresce, until the trap is again restored in a consecutive process. (b) the trapping pigment does not bleach but converts an associated molecule. Unless the latter conversion is restored in a consecutive reaction the next quantum cannot be used in photochemistry and will be re-emitted by the trapping pigment.

In either case, one can determine the number of traps by measuring the number of quanta required to raise the fluorescence from E_o to E_{max}. Assuming a quantum requirement of one per trapping molecule and absence of restoration reactions at 77°K, our measurements indicate a concentration of 1 trap per about ~50 chlorophyll molecules. Though still preliminary, our data probably show the correct order of magnitude; Kautsky et al. (2), observed at room temperature a similar but much faster initial rise of fluorescence and computed the presence of 1 quencher per ~400 chlorophylls. Using Porphyridium, Duysens and Sweers (3) arrived at a ratio 1:150. We indeed observed at 77°K for F700 (and probably F685 as well) a 10 times slower rise than at 300°K for F685.

This detrapping at low temperature indicates a photochemical phenomenon probably correlated with a primary act of photosynthesis. Since as far as we know no corresponding color change accompanies this detrapping, the second of the two mechanisms discussed above might be involved.

The number of traps in this system is much (~10x) greater than expected on the basis of the classical photosynthetic unit. Under appropriate conditions the rise time of F685 at room temperature reveals the same small photosynthetic unit. A trapping pigment present in so high a concentration (~2-5% of Chl.) might be detectable by rather direct methods.

In the following we will discuss some further observations concerning long wave absorption and emission bands which could

be related to it.

II. Correlation between P700, C700 and the low temperature emission bands.

With leaves at 77°K Butler (4) observed a band at 705 mu in the absorption spectrum as well as in the excitation spectrum of fluorescence. He assumed (a) that this pigment "C705" was the emitter of the strong 730 mu fluorescence band found earlier by Brody (1) at 77°K and (b) that C705 was identical to the long wave chlorophyll "P700" functioning as the trapping center in the long wave photoact (system I) of photosynthesis.

Identity of P700 and C705 appeared unlikely because the data indicated a concentration of C705 as high as 2-5% of total chlorophyll, whereas we never observed P700 in a concentration higher than 1 per 300 or 400 chlorophylls (5). An identification of P700 with F730 did not appear likely either: The photochemical bleaching of P700 is irreversible at 77°K and a fluorescence emission would have to come from its oxidized form. The possibility is not excluded, however, that P700 ox still does absorb, viz., at 690 mu (6). The following experiments bear on these questions:

Expt. Fig. 2 shows emission spectra (77°K) of chloroplasts briefly treated with increasing concentrations of acetone in water (7). The data show that a low concentration of acetone (20%) decreases the 730 mu fluorescence relatively less than the short wave bands. Higher concentrations produce band

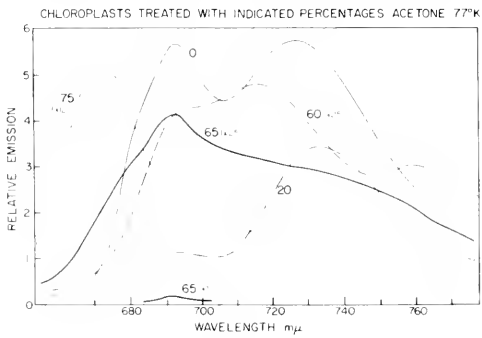

Figure 2

shifts and additional emissions in the long wave region, and also lower the yield severely. (The numbers in brackets indicate the factors applied to match the curves.) 75% Acetone

removes practically all chlorophyll and P700 and the extracted material shows an emission spectrum identical to that of a dilute chlorophyll solution. Treatment with 65-70% acetone, which barely yields a loss of P700, results in material which shows relatively less fluorescence at 730 mu and relatively more at 685 and 698 mu. As was to be expected, the emission of such preparations is not affected by the redox state of P700. The data of Fig. 2 indicate a closer correlation of P700 with the two short wave bands than with the long wave emission(s).

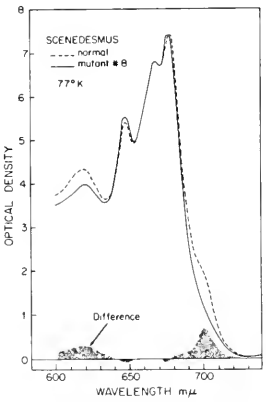

Figure 3

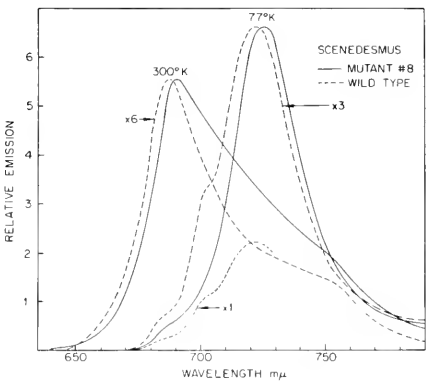

Figure 4

Figures 3 and 4 show data obtained with normal Scenedesmus cells and mutant #8 of Dr. N. Bishop. This mutant is capable of performing the quinone Hill reaction but not photosynthesis and photoreduction. Weaver and Bishop (8) noticed that it lacked the light induced fast EPR signal, according to Beinert, et al., (9) due to the oxidized form of P700. We indeed, did not find a light induced turnover of P700 in this mutant or extracts prepared from it. The difference between the two absorption spectra in Fig. 3 shows a distinct band at 700 mu amounting to $\leq$10% of the absorbance at 678 mu. This confirms Butler's (4) and Brown and French's (10) earlier observations of long wave absorption bands. One is tempted to identify "C700" with the small amount ($\sim$5%) of long wave "oriented" chlorophyll which will be discussed in this symposium by Dr. R. Olson (cf. also 11). The high concentration of C700 and its failure to undergo reversible photobleaching, argue against its identity with photocatalyst P700. On the other hand, the ability of the mutant to evolve oxygen [be it at lower than normal rate (8)] despite the virtual absence of C700 suggests that both pigments are part of photosystem I.

One could speculate that (some 20 molecules of) C700 function as energy collectors for (one molecule of) P700. Absence of C700 in the mutant then would explain the lack of a light induced P700 bleaching and EPR signal--energy transfer being impeded. (This hypothesis still allows the presence of P700 in the mutant--which remains to be proven).

An alternate hypothesis identifies C700 with P700: One could conceive that one of the oriented C700 molecules upon excitation loses an electron to primary acceptor (X) and regains it from cytochrome f or plastocyanin (6). If X, cyt. f and P.C. were only present in 1/20 the concentration of C700 (1/400 chl.) one would observe at any time only one "P700" per 400 chlorophylls. This hypothesis would fit sensibly with a rigid orientation of C700 around the reaction loci (11).

III. Sensitization of the various emission bands.

Figure 4 shows the fluorescence emission spectra of the two types of Scenedesmus. At room temperature the 685 fluorescence of the mutant is as high or higher than that of the normal cells (even if the latter are poisoned with DCMU to stop energy flow in order to obtain comparable conditions). We are not certain whether the anomalous emission between 700 and 760 mu in Fig. 4 is typical. At 77°K the mutant fluoresces stronger than the wild type at 730 mu, relatively weak at 685 mu, and the 698 band is practically absent.

Figures 3 and 4 show a distinct correlation between C700 and the fluorescence at 698 mu: both are practically absent in the mutant. The simplest explanation is that C700 is the emitter of F700.

The data, furthermore, indicate that the (77°K) fluorescence at 730 mu does not directly originate from either C700 or P700; F730 is high in the mutant lacking C700. It actually appears as if in the normal alga, C700 functions as a quencher for F685 at room temperature and possibly for F730 at 77°K. Quenching at room temperature could at least partly be an indirect effect: if C700 and/or P700 operated in photosystem I they would provide substrate for photosystem II, e.g. "Q_{ox}" in (3). However, quenching of F730 at low temperature must be due to a competition for absorbed quanta between C700 and "C730" (the chlorophyll responsible for F730).

We now meet a difficulty: Butler (4) observed, and we confirmed, that in leaves, chloroplasts, and green algae (where F730 is found at 716 mu) the sensitization of F730 shows a

maximum at 700-705 mu. This 700 maximum is quite weak in mutant #8 and subtraction of the excitation spectra measured with the two algae (as done in Fig. 3 for the absorption spectra) yields a distinct band at 698 mu, present in the normal alga only. Obviously C700 sensitizes the emission at 730 mu. This might still be compatible with a quenching of F730 if one assumes that C700 transfers energy from sensitizing pigment (Chl. a) to P730, but either can be bypassed or is needed only in a low concentration such as still might be present in the mutant:

$$h\nu \longrightarrow \text{Chl a} \longrightarrow \text{C700} \overset{\text{F700}}{\nearrow} \longrightarrow \text{C730} \overset{\text{F730}}{\nearrow} \qquad (1)$$

In the previous section we have tentatively located C700 in photosystem I, if scheme (1) were correct, F730 should be sensitized by the same pigment system. To check this, we have measured the excitation spectra for F700 and F730 in chloroplasts and various types of algae. In each species these spectra proved to be quite similar for the two emissions--which is an argument for scheme (1). However, the data failed to indicate a correlation with "photosystem I":

In Anacystis the excitation of F700 and F730 revealed the typical ineffectiveness of 680 mu light (1/2 - 1/3 of 630 light) and also lacked the 700 band which is so evident in chloroplasts and green algae. (The red alga TX 27, however, showed a distinct sensitization band at 710 mu.)

Figure 5 shows the fluorescence emission of the alga TX 27 excited either by green light (546 + 578 mu) which sensitizes photosystem II (and I) via phycoerythrin or by blue light (436 mu) which mainly sensitizes photosystem I.

The multiplication factors required to bring the spectra of Fig. 5 to equal height (indicated on the curves) show that green light is much more effective than blue, both at room temperature (4x) and at 77°K (16x). Although, at 77°K the 730 band is predominant in the blue excited emission, it still is induced (9x) more effectively by green light. For the 685 and 698 emissions this ratio is ~50 fold. The data of Fig. 5 [in accord with those of Brody and Brody (1)] do not support the simple thesis that "F730" originates from photosystem I. They further show that F700 as well as F685, even more exclusively than F730, are sensitized by green light i.e. by photosystem II. Actually, also the excitation spectra measured with green cells--although more difficult to analyze--favor this conclusion.

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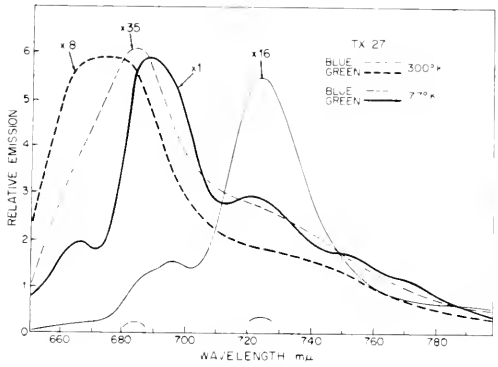


Figure 5

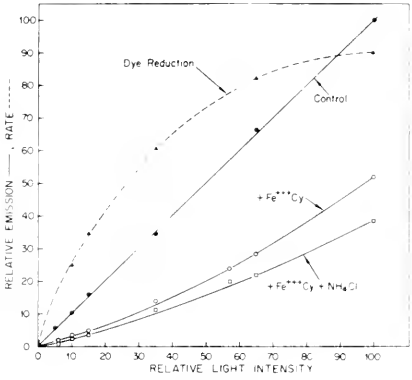


Figure 6

IV. Fluorescence yield and electron transport.

Figure 6 shows measurements (made at room temperature) of fluorescence as a function of intensity, run concurrently with measurements of the rate of dye reduction by fresh chloroplasts. In confirmation with the earlier analysis of Lumry et al. (12), the data show that efficient electron transport corresponds to a low yield of fluorescence. In the absence of a Hill oxidant the yield is high at all intensities. In the presence of ferricyanide the yield is low in weak light. Addition of NH_4Cl--which by uncoupling phosphorylation accelerates the rate by about a factor two--lowers the fluorescence yield even further (to 25% of the control value). In higher intensities electron transport approaches its saturation rate and the fluorescence yield rises again.

Effect of Subsequent Additions upon the Fluorescence Yield of Fresh Chloroplasts in Weak Light

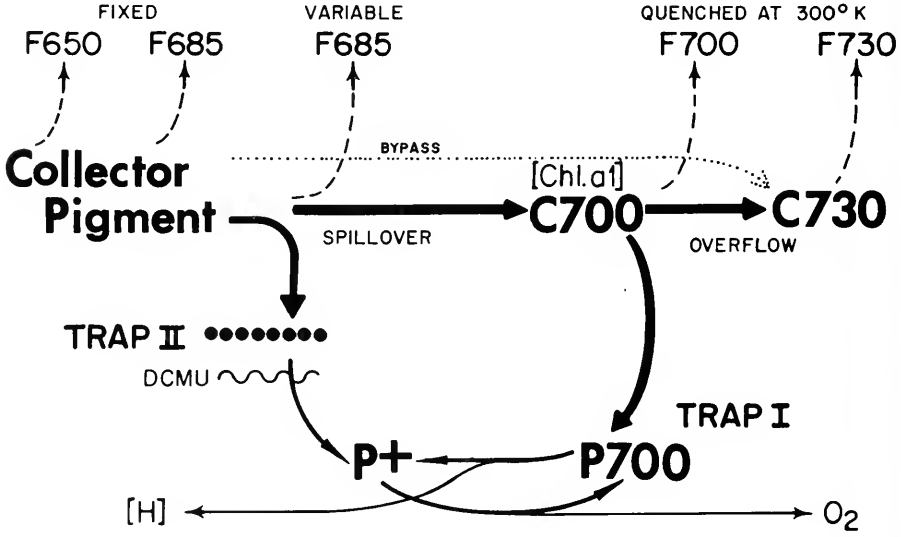
Addition	Rel. Yield
None	100
PPNR (Saturating)	98
10^{-4}M TPN	52
10^{-5}M DCMU	86
10^{-4}M DCPIP + Ascorbate	82

The above table shows that addition of PPNR (which does not induce significant electron transport), has no effect, whereas successive addition of TPN lowers the yield. Addition of DCMU again brings the fluorescence close to the control value--indicating that electron transport stops. A

subsequent addition of indophenol dye and ascorbate, which restores the photoreduction of TPN does not restore the suppression of fluorescence. We may assume that the photoreduction of TPN in the presence of DCMU and reduced dye requires only the long wave photoreaction (6). Although this conversion can occur with a high quantum yield, (13) it is not reflected in a suppression of fluorescence.

DISCUSSION

The following scheme seems to satisfy most of the presented data. It assumes that the bulk of the light absorption is carried out by one pigment assembly only. Some fluorescence escapes at all times from the partners of this collector system. Absorbed quanta drain preferentially in trapping molecules II which occur in a concentration of 1 per 50 Chl. (cf. Fig. 1). As long as these traps are unexcited (and kept in the receptive state by a dark reaction) only the accidental (fixed) fluorescence occurs.



After a trap II is photoconverted, however, the next excitation will flow on to the long wave pigment C700 ["spill over" (16)]. Since the coupling with C700 is rather weak, this transfer competes with an additional ("variable") fluorescence emission of the collector system [cf. Franck (17)]. If all absorbed quanta flow towards C700 (as in the absence

of substrate or the presence of DCMU which blocks alternate path II), the variable fluorescence will be maximal, although system I might still operate at full efficiency (cf. Fig. 6, Table 1).

Secondary collector C700 transfers its excitations to the final trap of "photosystem I": P700. Upon excitation P700 produces a strong reductant ("H") and is left behind as a weak oxidant. Trap II, after its photoconversion, is capable of reducing $P700^+$ and producing O_2. Except for the fact that it should be (associated with) a chlorophyll type pigment, the chemical nature of this trap and its mode of dark conversion are immaterial for the present discussion.

The data indicate that in green plants C700 and trap II occur in about equal concentration--but they seem to exclude the identity of the two compounds. [System II can operate in the absence of system I (cf. Section II)].

A peculiar feature of this scheme is that the "primary collector unit" amounts to only 50 pigment molecules--a situation also found in bacterial photosynthesis (18). For optimal operation, each unit should, on the average, transfer quanta to T II and C700 in the proper ratio (e.g. 1:1), but since the mechanism is self-regulating it does not require a definite ratio between T II and C700 or an association of these with a distinct group of collector pigment. This self-regulation is effected by the connecting electron transport system mediated by P700--fed at both ends by some 10 small units. The result of this "double focussing" is the classical unit of 400 chlorophylls.

The scheme provides for a second "switch" in the transfer chain: quanta which fail to find P700 can either be degraded in C700 itself, [at 77°K re-emitted as F700 (cf. Fig. 4)] or escape to C730--which also emits only at low temperature. (Long wave light does not yield fluorescence at 300°K.) The fact that at 77°K a considerable fraction of the absorbed light (50%) can be re-emitted at 730 mu (cf. Fig. 5) suggests that this is a functionally significant process. One can ascribe an important task to pigment C730: the harmless degradation of excess quanta which might otherwise lead to photoinhibition.

In green plants (C700) amounts to only 5-10% of (Chl.) and causes a decline of the quantum yield of O_2 evolution only beyond 690 mu: Due to the impossibility of reversed quantum flow traps II are not excited by light absorbed by C700

itself.

In blue-green algae, however, even 680 mu light is used ineffectively. We therefore, assumed that in these algae C700 is replaced (or amplified) by a significant fraction of the total chlorophyll [indicated (Chl. a 1)].

ACKNOWLEDGEMENTS

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Bessel Kok

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LIGHT-DRIVEN CYTOCHROME REACTIONS IN ANACYSTIS AND EUGLENA

John M. Olson and Robert M. Smillie

The basic similarity in cytochrome physiology between Anacystis nidulans and Euglena gracilis, strain Z, is impressive in view of their gross dissimilarities in size, structure, and pigment content. We have investigated the cytochrome reactions by sensitive spectrophotometric methods in order to gain some insight into the patterns of energy transfer from the various light receptors to the reaction centers involved in the two photochemical reactions of green plant photosynthesis and also to elucidate the pathways of photosynthetic electron transfer. The major thrust of this presentation will be the implications of experiments on whole cells in which both wavelength and intensity of monochromatic actinic light have been systematically varied. Some preliminary observations of the effect of carbonyl cyanide-m chlorophenylhydrazone (CCCP) are presented, and the light-driven reduction of cytochrome b_6 in Euglena chloroplast fragments is described.

INTACT ALGAE

Two Light Reactions: The evidence for two essential light reactions which most clearly laid the precedents for the present work was that obtained by Kok[1], Witt[2], and Duysens[3]. The light-induced oxidation of c- or f-type cytochromes in green plants was clearly established, and the light-driven reduction by a second photoreaction was demonstrated. We have confirmed the observations by Amesz and Duysens[4] of light-driven cytochrome reactions in Anacystis and have identified the major component to be cytochrome f-555 on the basis of the alpha trough at 556 mμ in light-minus-dark difference spectra. In Euglena the high potential cytochrome-552 reacts to light. The effects of the two photochemical reactions on these f-type cytochromes are illustrated in Figure 1. In both algae far-red light causes a rapid oxidation to the steady-state level; but light below a certain critical wavelength causes an initial rapid oxidation followed by a slower reduction to the final steady-state when the proper intensity is used. The diphasic kinetics disappear as the light intensity is lowered as shown in Figure 2. The curves ("initial peak" and "steady-state") for 0.62 μ light in Figure 2 are

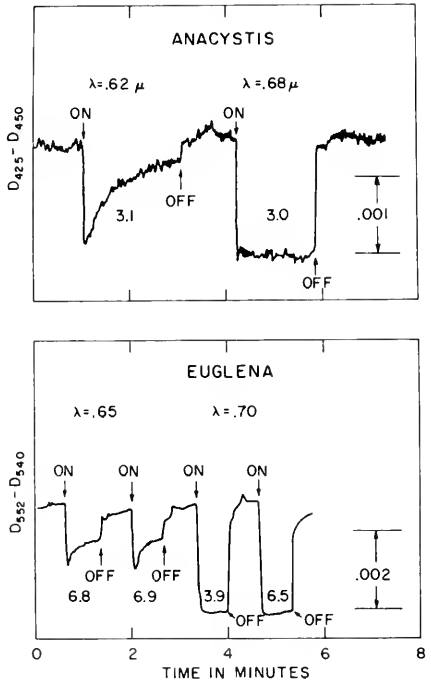

Fig. 1. Kinetics of cytochrome oxidation and reduction in Anacystis and Euglena. Actinic light intensities are indicated by the numbers below or inside each ON-OFF reaction tracing. Units are 10^{-9} einstein cm^{-2} sec^{-1}.

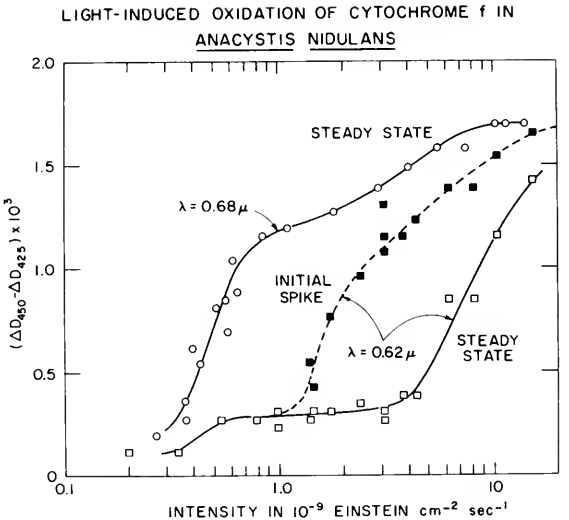

Fig. 2. Steady-state level of cytochrome oxidation versus intensity at two wavelengths of actinic light. The initial spike curve for light of 0.62 μ indicates the maximum initial change upon illumination as shown in the left hand traces of Fig. 1.

typical in shape for those wavelengths which activate both photochemical reaction 1 (R_1, the cytochrome-oxidizing reaction) and reaction 2 (R_2, the cytochrome-reducing reaction). The steady-state curve for 0.68 μ light is typical for those wavelengths which activate R_1 only. Completely analogous steady-state curves have been obtained with Euglena using 0.70 μ light for R_1 and 0.65 μ light for $R_1 + R_2$. Kok and Beinert[5] have obtained from Anacystis cells curves of ESR signal intensity (ascribed to photocatalyst P-700) vs. actinic light intensity which are very similar to our cytochrome curves.

Action Spectra: The relative effectiveness of the various light-absorbing pigments in driving R_1 and/or R_2 can be ascertained in principle by determining the action spectra for light-driven cytochrome oxidation and for light-driven cytochrome reduction. We have derived action spectra from families of curves of cytochrome oxidation vs. actinic light intensity. In

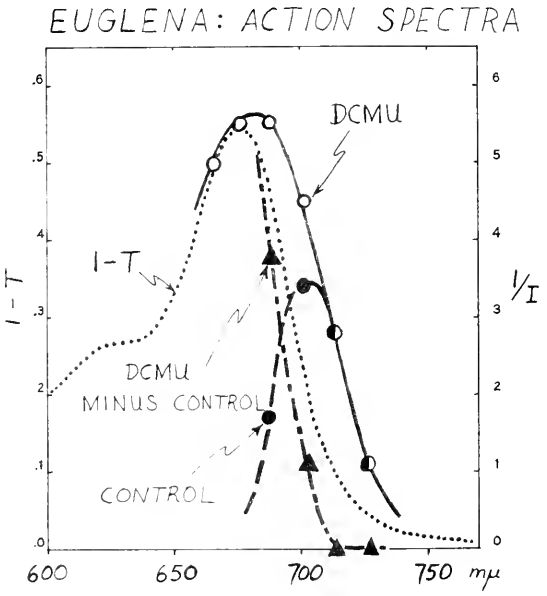

Fig. 3. Action spectra of cytochrome-552 oxidation in arbitrary units of reciprocal intensity compared to the absorption spectrum (1-T) of the whole cell suspension.

Anacystis the action spectrum of R_1 was obtained by cooling the cells to 2° C at which temperature the rate of cytochrome reduction by R_2 is negligible. Chlorophyll a was found to be 40 to 70 per cent more effective than phycocyanin in R_1, as was found by Amesz and Duysens[4]. We also confirmed the finding of Amesz and Duysens that chlorophyll a is also effective in R_2, although much less than is phycocyanin. In Euglena, the action spectrum of R_1 was obtained by adding 3-(3,4 dichlorophenyl)-1,1-dimethylurea (DCMU), 1.5 x 10^{-5} M, to the cell suspension (Fig. 3) in order to block R_2. Comparison of the action spectrum to the absorption (1-T) spectrum indicates that chlorophyll a is effective in R_1, but that a pigment with an absorption peak at about 705 mμ is considerably more effective. This pigment probably corresponds to Kok's photocatalyst P-700[1] and Butler's C-705[6]. When the action spectrum of cytochrome oxidation (steady-state) is determined in the absence of DCMU, both R_1 and R_2 determine the curve marked CONTROL in Figure 3. The absorption spectrum of those pigments which are effective in both R_1 and R_2 is obtained by subtracting the CONTROL curve from the DCMU curve. From Figure 3 it can be seen that chlorophyll a is effective in R_2 as well as in R_1. Chlorophyll b apparently does not play a unique role in sensitizing R_2 in Euglena, as has been suggested in other instances of green plant photosynthesis[7,8].

Light Reaction No. 1: After illumination of Anacystis or Euglena, the cytochrome(s) oxidized in the light return to the reduced state in the dark, even under conditions when R_2 is totally inoperative. Certain characteristics of the cytochrome response to R_1 suggest that reducing equivalents formed in R_1 can reduce oxidized cytochrome(s) via a temperature sensitive pathway. The temperature sensitivity of the cytochrome reductase activity can be seen from the increase in the half time for the "light-off" cytochrome reduction as the temperature is dropped. In Euglena, for example, $t_{1/2}$ increased from ca. 6 sec at 25° to ca. 26 sec at 2°. The temperature-independence of the "light-on" cytochrome oxidation can be seen from initial rate measurements and from the lower light intensity required to maintain a given level of cytochrome oxidation at lower temperature. In Anacystis, for example, the relative intensities of 675 mμ light required to give half maximal cytochrome oxidation at 2°, 20°, and 37° were 1, 4, and 10, respectively. Evidence for a cyclic flow of electrons via a cytochrome system in R_1 is provided by the kinetics of the "light-on" and "light-off" cytochrome responses at very low light intensities in both algae. As shown in Figure 4, the oxidation reaction does not become apparent immediately upon illumination with very low intensity; a definite lag occurs. The

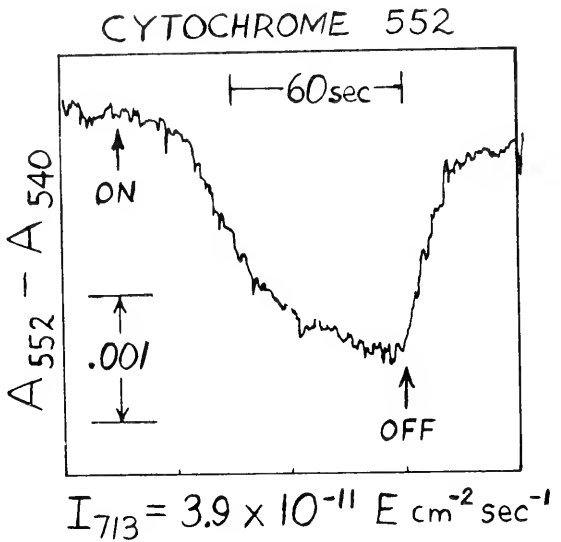

Fig. 4. Kinetics of cytochrome-552 oxidation and reduction with very low intensity far-red light at room temperature in the presence of 4 x 10^{-6} M DCMU.

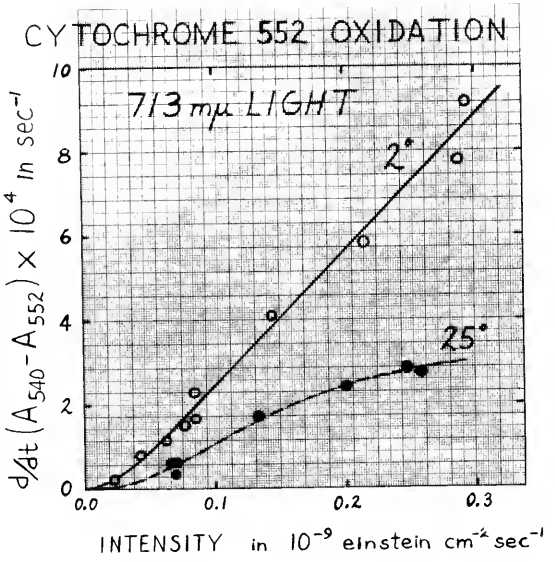

Fig. 5. Maximum net rate of cytochrome-552 oxidation versus intensity of far-red light at 2° and 25°. The sample absorption at 713 mμ is about 30 per cent.

maximum net rate of oxidation during the "light-on" phase is much less than the initial rate of reduction observed in the "light-off" phase. A simple explanation of the "sluggish" cytochrome oxidation is that the rate of cyclic electron flow is rapid enough at room temperature to compete with the oxidation reaction at low light intensity. The maximum net rate of oxidation upon illumination is not proportional to intensity at these very low intensities; the rate vs. intensity curve is sigmoid as shown in Figure 5. As the temperature is dropped, however, the rate curve more nearly approaches a straight line.

Quantum Requirements: Preliminary estimates of the quantum requirement for cytochrome-552 oxidation in Euglena range from 2 to 8 quanta per electron based on rates of absorbancy change upon illumination. In Figure 5 the slope of the rate curve at 2° indicates a quantum requirement of 2 if $\Delta\epsilon_{552} - \Delta\epsilon_{540}$ is assumed to be 2×10^4 M^{-1} cm^{-1}. The lowest observed values are substantially lower than the estimate of 7-10 for Anacystis(4).

Cytochrome b Reactions: Light-induced reactions of cytochrome b in whole cells are observable only under special circumstances. Under physiological conditions cytochrome f-555 is the main pigment to respond in Anacystis; sometimes a slight response of the low potential cytochrome C-550 is also observed. In Euglena, only the high potential cytochrome-552 is observed. Cytochrome b oxidation in Anacystis caused by far-red light (0.70 μ) can be observed in addition to cytochrome f oxidation when cells are cooled to 2° C or when cells are permitted to become anaerobic at room temperature. In both Anacystis and Euglena, the addition of 5×10^{-5} M CCCP permits the light-induced oxidation of cytochrome b with far-red light (R_1 only) either with or without the oxidation of f-type cytochrome (Fig. 6). In Anacystis the cytochrome b oxidation is superimposed on the usual cytochrome f + c oxidation. In Euglena, the cytochrome b oxidation appears to replace cytochrome-552 oxidation initially, but the cytochrome-552 light reaction reappears almost completely after 40 min without appreciable change in the cytochrome b reaction. The mechanism of CCCP action on photosynthetic electron transfer is not known.

An attempt to demonstrate the light-induced reduction of cytochrome b by R_2 in whole cells (Euglena) indicated a possible transient small increase in reduced cytochrome upon illumination with high intensity red light (0.65 μ). The steady-state change was, however, either zero or a slight net oxidation.

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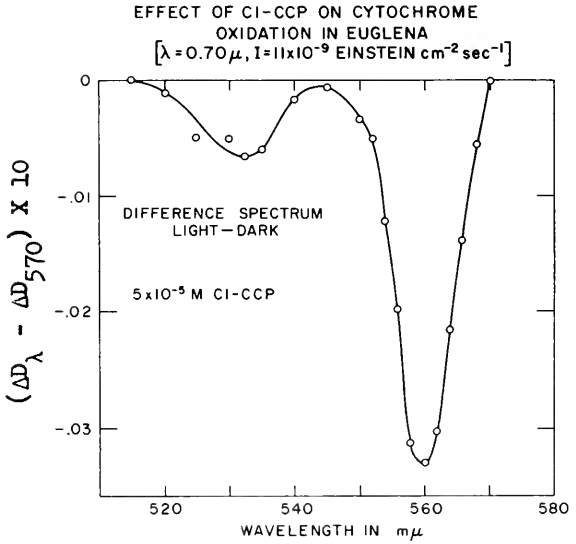

Fig. 6

EUGLENA CHLOROPLAST FRAGMENTS

Cytochrome b_6 Reduction: When washed chloroplast fragments are prepared from Euglena cells in late log phase, most of the cytochrome-552 is lost. When such fragments are suspended in .025 M Tris, pH 7.8 with 10^{-3} M $MgCl_2$, strong light causes a gradual reduction of cytochrome b_6 which remains bound to the fragments (Fig. 7). If the light is turned off after the reduction is complete, the reoxidation in the dark is extremely slow. If, however, the light is kept on, and the intensity dropped a factor of 10, a light-induced oxidation of the cytochrome occurs after an initial lag of about 20 sec (see upper left insert in Fig. 7). The observation of a light-induced reduction or a light-induced oxidation of cytochrome b_6 depending upon light intensity and the redox state of the cytochrome suggested that both R_2 and R_1 were still functioning. The absence of significant oxidation (or reduction) of cytochrome b_6 in the absence of light indicated that this cytochrome cannot be merely a component of an electron transfer system which "short-circuits" a single photochemical reaction.

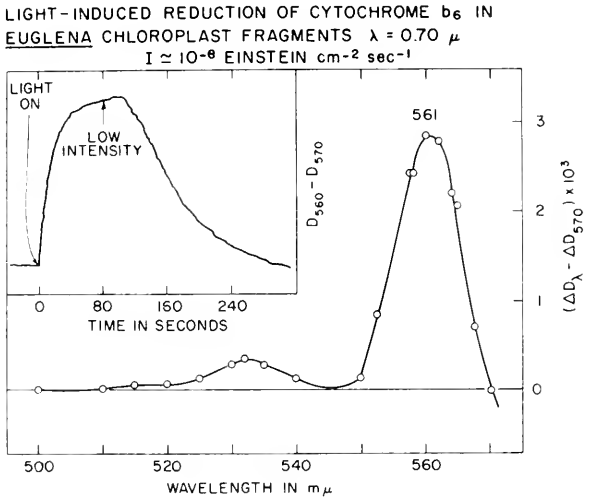

Fig. 7

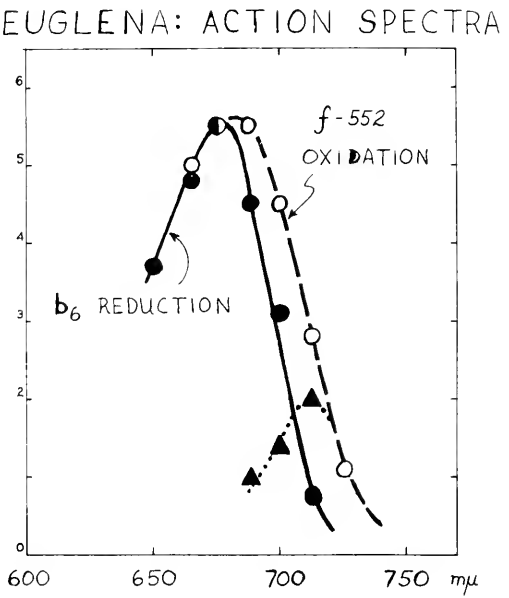

Fig. 8. Action spectra: Cytochrome-552 oxidation in whole cells poisoned with 1.5 x 10^{-5} M DCMU and cytochrome b_6 reduction in chloroplast fragments. The two spectra have been normalized at 675 mμ and the difference spectrum plotted (triangles and dotted curve).

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The action spectrum of the light-induced reduction of cytochrome b_6 in chloroplast fragments is compared to the action spectrum of cytochrome-552 oxidation in whole cells poisoned with DCMU in Figure 8. The former spectrum corresponds to the action spectrum of R_2, and is further evidence that chlorophyll a is effective in R_2. The difference between the normalized spectra for R_1 and R_2 indicates again that a far-red pigment analogous to P-700 is active in R_1, but not in R_2.

Effect of 1,10 Phenanthroline: This inhibitor (10^{-3} M) decreased the steady-state level of cytochrome b_6 reduction during illumination to about 10-20 per cent of the total change observed in the absence of inhibitor. In addition, a rapid reoxidation of the cytochrome b_6 took place immediately when the light was turned off. Therefore, no light-induced oxidation of cytochrome b_6 at low intensity could be observed in the presence of 1,10 phenanthroline. It appeared that the cytochrome b_6 was now part of a "short circuit" of R_2.

Interaction of Cytochrome-552: When purified cytochrome-552 (ca. 10^{-6} M) from Euglena is added to washed chloroplast fragments (ca. 10^{-4} M chlorophyll), illumination causes the oxidation (or reduction) of the added cytochrome-552. High intensity illumination causes the oxidation of cytochrome-552. When the light is turned off, a rapid reduction occurs in the dark with a half time of ca. 6 sec. (The light-induced reduction of cytochrome b_6 which is concurrent with the oxidation of cytochrome-552 is about 1/3 the change observed in the absence of added cytochrome-552.) Very low intensity illumination causes a relatively slow oxidation of cytochrome-552, but the final extent of oxidation is considerably greater than that observed with high intensity. Furthermore, no reduction is observed upon cessation of the light. A light-induced reduction of cytochrome-552 can be demonstrated with high intensity illumination after partial oxidation with low intensity light.

Addition of 5×10^{-4} M 1,10 phenanthroline to the reaction mixture slows down the reduction of cytochrome-552. The light-induced oxidation at high intensity is at least doubled and the subsequent reduction in the dark is slowed to about 1/10th the rate in the absence of inhibitor.

SUMMARY AND CONCLUSIONS

1) There is not necessarily a one-to-one correspondence between a given light-absorbing pigment (e.g. chlorophyll a) and a

given light reaction (e.g. R_1). In either Anacystis or Euglena, a photon absorbed by chlorophyll a has a significant probability of driving R_2 instead of R_1. Analysis of energy transfer pathways should be made in terms of relative probabilities of energy transfer to R_1 and R_2 from a given light absorber.

2) The cytochrome oxidation vs. intensity curve (Fig. 2) for actinic wavelengths which activate both R_1 and R_2 suggest that the transfer ratio R_1/R_2 may increase as the light intensity is raised to very high levels. This implies that R_2 approaches light saturation at lower intensities than R_1.

3) Cytochrome f (or -552) is oxidized by R_1 via a mechanism relatively insensitive to temperature, and is reduced by R_2 and/or a short-circuit pathway around R_1. Cytochrome-552 oxidation apparently requires 2 quanta per electron. Cytochrome reduction has a temperature coefficient of about 2 per 10 degrees.

4) Light-driven reduction of cytochrome b_6 in Euglena chloroplast fragments and the interaction of added cytochrome-552 supports the proposal of Hill and Bendall[9] that electron flow from R_2 to R_1 is mediated by cytochrome b_6 and cytochrome f (or -552).

5) The mechanism of 1,10 phenanthroline inhibition of the Hill reaction and photosynthesis may be linked to the apparent short-circuiting of R_2 via cytochrome b_6 in chloroplast fragments.[10]

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THE TEMPERATURE INSENSITIVE OXIDATION OF CYTOCHROME F IN GREEN LEAVES - A PRIMARY BIOCHEMICAL EVENT OF PHOTOSYNTHESIS

Britton Chance and Walter D. Bonner, Jr.

The temperature insensitive oxidation of a cytochrome component adjacent to chlorophyll has proved to be an incisive tool in the study of the basic mechanisms of primary photo-reactions at the biochemical level. In the context of this paper the "primary" light-induced reaction is that which involves a chemical step, and therefore we exclude the physical processes by which chlorophyll may become activated.

A number of aspects of the temperature insensitive oxidation of cytochrome c in the purple sulfur bacteria Chromatium have been summarized elsewhere (1), and special attention has recently been paid to the fact that the reaction is not only temperature insensitive but is also viscosity insensitive (2). The application of this method to green plants has not previously been very fruitful. While H. T. Witt has recorded the low temperature oxidation of cytochrome f, the observation has been restricted to chloroplast suspensions and, in fact, to chloroplast suspensions which are specifically treated with sucrose in order that the effects be observed (3). As yet no observations have been made of the low temperature response of cytochrome f in the intact leaves of green plants, although numerous observations have been made at room temperature in leaves and in suspensions of algae; for example, Chlorella (4), Anacystis (5) and Porphyridium (6) and in chloroplasts (7).

This paper describes the application of the double-beam spectrophotometer to the detailed quantitative study of cytochrome f oxidation at 77° K in a variety of leaves. The experimental result allows an evaluation of the role of cytochrome f oxidation in relation to the "P_{700}" absorption band. In addition, we provide an accurate representation of the relationship between light absorption in chlorophyll a and the rate of cytochrome f oxidation in leaves.

Materials.

The materials used were market spinach leaves, leaves plucked

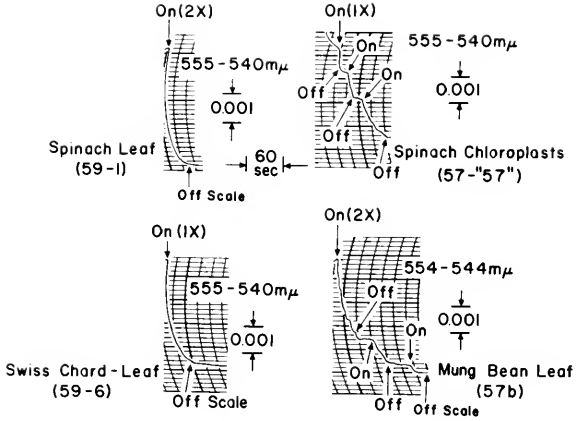

Figure 1. Light-induced oxidation of cytochrome f in three types of leaves and one preparation of chloroplasts. 680 mμ actinic light; a downward deflection corresponds to a decrease at 555 or 554 mμ measured with respect to the reference wavelength (540 or 544 mμ). Light intensities employed relative to each other are indicated by the symbols "1X" or "2X". Experiment 59, 1 - 4.

Comparison of Cytochrome f kinetics

in different leaves and in chloroplasts

at 77° K

Material	λ	ΔOD⁺	10^4 ΔOD⁺/sec	Intensity*	10^4 ΔOD⁺/sec/ unit intensity	Expt.
Spinach leaf (market)	555-540	0.0069	0.18	1.5	0.12	57a-1
Spinach leaf (fresh)	555-540	0.0045	0.55	3.3	0.17	59 -1
Spinach Chloroplasts	555-540	0.0050	0.90 0.25	3.3 1.0	0.27 0.25	57a 58 57a 59
Swiss Chard leaf fresh	555-540	0.0054	0.59	1.5	0.39	59 6
Mung bean leaves (etiolated)	554-544	0.0044	0.35	3.3	0.11	57a-55

⁺ per leaf thickness except for chloroplasts (1mm path, 0.279 mg/ml)

* Arbitrary units

I

TABLE I

directly from young spinach and Swiss chard plants, and chloroplast preparations (kindness of Mr. Stephen Sikes). Etiolated Mung bean leaves were obtained directly from the plant and were greened for several hours with the red light prior to use. The properties of these leaves have been reported on in more detail by Dr. Bonner (8). The leaves are held lightly between two lucite plates. These preparations were rapidly frozen by plunging them into liquid nitrogen in an aluminum holder of small specific heat. Occasionally, the leaves crack at low temperatures, especially if they are held too tightly by the lucite plates.

The leaf absorbancies are very high, not only due to their chlorophyll content (0.3-0.4 mmoles/kg wet weight) but also due to the non-specific light absorption of the leaf. The effective transmission in this apparatus is 1.6 per cent at 700 mμ and even less at shorter wavelengths. Thus, nearly complete absorption of the incident light occurs.

An example of the responses of these four types of biological materials is given in Fig. 1 where we display on a compressed time scale the optical density decreases at 555 mμ measured with respect to a nearby reference wavelength (540 or 544 mμ). In each one of the four records, it is seen that the optical density change of the order of approximately 0.005 is obtained. It is seen that a rapid downward deflection of the traces (corresponding to a decreased absorbancy at 555 mμ) is obtained when the actinic light (680 mμ) is turned on. In each case, the leaf thicknesses were between .4 and .6 mm. Two levels of actinic intensity are employed (1X and 2X). In the case of the chloroplast suspension and the Mung bean leaves the actinic light is interrupted periodically and the oxidation is seen to come to a halt. Thus, the effects of the measuring light are not objectionable under the experimental conditions. However, small effects can be observed in the chloroplast suspension.

Table I summarizes a number of properties of the cytochrome f responses in the four types of leaves and the one type of chloroplast preparation studied. As indicated in the Table, the optical density changes for the leaf thickness are in the range of 0.044 (Mung bean leaves) to 0.0069 for market spinach leaf. The rates of cytochrome f oxidation, which will be discussed in more detail below, when put on a basis of an arbitrary unit of light intensity, are of a similar order of magnitude although the fresh Swiss chard leaf seems to excel the other biological materials, even the chloroplast preparation.

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EXPERIMENTAL METHODS

A Dewar flask is attached onto the emergent light fitting of the double-beam spectrophotometer (9, 10). The one inch photo-multiplier is attached directly to the housing of the Dewar flask. Since this Dewar has right-angle windows, side illumination is readily obtained from either a tungsten lamp (680 mμ illumination) with appropriate interference filter or a mercury arc (436 mμ illumination) with a multielement filter (Eppendorf). The cuvette is held in the picture and contains an aluminum block with a pair of lucite plates between which the leaf is gently pressed. A small mirror serves to reflect the actinic light upon the leaf, out of the way of the measuring light. All other aspects of the double-beam spectrophotometer are as in previous communications (11). The levels of actinic illumination are low; since cytochrome f responds as a "quantum counter", high levels are not required to cause maximal oxidation as they are at room temperature. We are not reporting quantum efficiencies for cytochrome f oxidation in this paper and therefore light intensities are reported in arbitrary units. The values are about 10^{-10} to 10^{-11}/ Einsteins/cm^2/sec.

When 436 mμ excitation is employed, the rate of cytochrome f oxidation is slower than with 680 mμ excitation, as indicated in the Figures below. However, the extent of the reaction is the same, since the reaction is essentially irreversible.

Absorbancy enhancement.

With cytochrome c the absorbancy increment in the oxidized minus reduced spectra at 549 mμ is 4.5 times greater. Presumably, such values apply to leaves, but detailed controls are necessary to ensure this. This factor is not known for P_{700}. For the purposes of this paper, we are emphasizing the ratio of the rates at 555 and 705 mμ. This comparison may be made by assuming the same enhancement for the two wavelengths without the need for its absolute value.

Effect of the measuring light.

At low temperatures, cytochrome f becomes effectively a "quantum counter" and therefore, prior illumination with undue intensities of excitation light will vitiate the desired response. We have, however, illustrated the effect of a high level of measuring light intensity in Figure 2. In this illustration, the measuring light is that obtainable from a 3 mμ slit of the Bausch

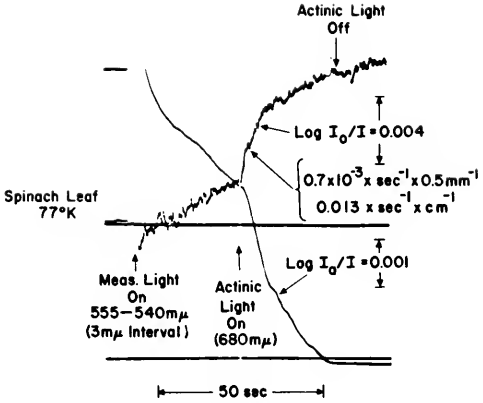

Figure 2. Illustrations of the kinetics of the light-induced cytochrome f oxidation under conditions where the measuring light is of sufficiently high intensity to cause rapid oxidation by itself. Time proceeds from left to right. The deflection of both traces with time indicates a decrease of absorbancy at 555 mμ with respect to 540 mμ. The sensitivities employed in the two traces are different and are indicated on the diagram. Response time of the two traces is also different; that of the lower sensitivity has a lower response time corresponding to less than 1/2 second, while the trace for the higher sensitivity has a response time of approximately 2 seconds. The moment of illumination with actinic light is indicated, and its wavelength is 680mμ. A spinach leaf employed in this experiment was a mature plant (market spinach). The rate of absorbancy change on illumination with actinic light is indicated per leaf thickness (0.5 mm) or per centimeter. Experiment 53 A 1.

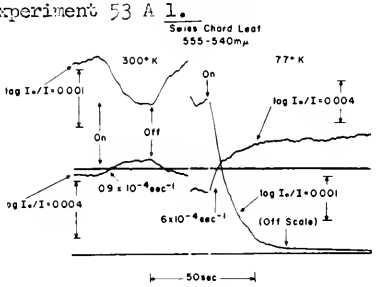

FIGURE 3

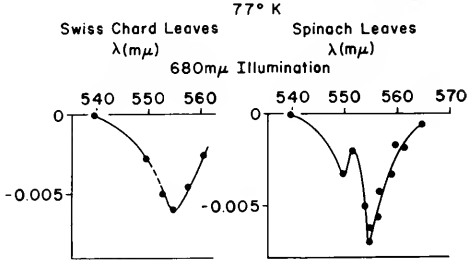

FIGURE 4

Figure 3. A comparison of the light response of Swiss chard leaf at 300° and 77° K. The convention in recording is similar to that of Figure 2; i.e., two traces of different sensitivities and response speeds. The actinic illumination is 680 mμ. Time proceeds from left to right; initial rate of absorbancy change is calculated per leaf thickness (0.4 mm). Experiment 59-6.

Figure 4. Low temperature difference spectra for Swiss chard and spinach leaves obtained with the double-beam spectrophotometer, according to the method illustrated by the preceding Figure. A number of similar leaves are selected and illuminated consecutively at low temperatures. The absorbancy changes are indicated per leaf thickness, 680 mμ actinic illumination. Experiment 62, 58.

and Lomb monochromators. Upon the moment of illumination of the sample, a steady state deflection of the two traces is observed which increases when the actinic light (680 mμ) is turned on. It is seen that the measuring light intensity is adequate to cause an appreciable rate of oxidation of cytochrome f and roughly half of the cytochrome f has been oxidized by the time the actinic light is turned on.

A criterion of satisfactory operation may be taken as the ratio of the response time of the spectrophotometer (0.3 sec for 10 to 90 per cent in this case) to the time for the measuring light to cause an arbitrary oxidation of cytochrome f (10 per cent is used here). This ratio, M, is over 40 here and may be useful in comparing different spectrophotometers.

With the actinic illumination employed here, a response time of 1 sec (10 to 90 per cent) is adequate in order to diminish the oxidation of cytochrome f prior to illumination. We have reduced the spectral interval from 3 to about 1 mμ. In most of the traces which we report here, the effect of the measuring light prior to illumination with actinic light is negligible; the ratio M is over 25, even when using 700 mμ as the measuring light.

The recording is usually made with double traces at different gains and at different response speeds. Thus, the rapidly responding trace (showing an upward deflection) is at a lower gain and at a higher response speed (1 sec), while the downward deflecting trace is at a higher gain an a lower response speed (2-3 sec). However, the respective upward and downward deflections of both traces correspond to a decrease of absorbancy at 555 with reference to 540 mμ.

In these studies in which the absorbancy changes due to P_{700} are measured, certain controls and precautions are observed to insure that fluorescence changes are not causing artifactual responses. First, it should be pointed out that the observations of Butler (12) on the fluorescence of chlorophyll show that fluorescence enhancement of approximately 20 per cent caused by light in the region of 620 mμ is decreased considerably by light of wavelengths of 700 mμ. Therefore, a fluorescence artifact would be in the opposite direction from the absorbancy decrease observed; i.e., 705 mμ illumination decreases the long wave fluorescence of chlorophyll relative to that of 635 mμ. Thus, with the double-beam spectrophotometric technique, the absorbancy change would not be confused with the fluorescence change, as they are in opposite directions. Secondly, the ability to record the kinetics on a fast

as well as a slow time scale permits the observation of the relatively instantaneous fluorescence change as separate from the kinetics of the P_{700} oxidation.

Steady state illumination allows the possibility of the time discrimination between the fluorescence and absorbancy changes as the fluorescence change would occur in a short time, while absorbancy changes, as clearly indicated in the charts, requires about half a minute to reach the steady state level. We have, therefore, reasonable assurance that the double-beam method guards against the very annoying fluorescence due to chlorophyll in emission, in the region of 700 mμ.

When the double-beam spectrophotometer is used with intense actinic illumination of fluorescent materials, where the average photocurrent may increase greatly during actinic illumination, it is necessary to connect the photomultiplier output directly to the ac amplifier, bypassing the chopper contacts used for calibration or for single-ended operation. However, a doubling of the average photocurrent causes no difficulties.

EXPERIMENTAL RESULTS

A comparison of the light induced kinetics at room and low temperatures.

Figure 3 illustrates room and low temperature kinetics of a Swiss chard leaf upon illumination with 680 mμ actinic light. This comparison is facilitated by the possibility of measuring the room temperature kinetics before filling the Dewar flask with liquid nitrogen. On the left hand portion are the room temperature kinetics which are seen first to be rather small in amplitude in comparison with the low temperature kinetics. (The rate of reaction is apparently more rapid at the low temperature in spite of the decrease of sensitivity to absorbancy changes at the low temperatures.) Two effects are undoubtedly involved. First, an enhancement of the absorbancy change due to increased light scattering and a sharpening of the cytochrome band occurs. Second, the dark reduction of cytochrome f which, at room temperature is almost identical in rate to the oxidation, does not occur, and hence, the correct velocity constant for cytochrome f oxidation can be obtained at the low temperature. This diagram suggests that measurements of quantum requirements for cytochrome reactions may be more accurately measured at the low temperatures (11)

Difference spectrum for illumination at low temperatures.

By choosing a number of leaves of similar size and hence thickness, it is possible to repeat the experiment of the previous Figure 3 at various wavelengths of measuring light and to obtain

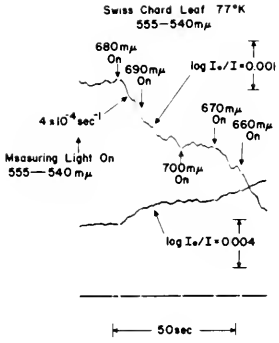

FIGURE 5.

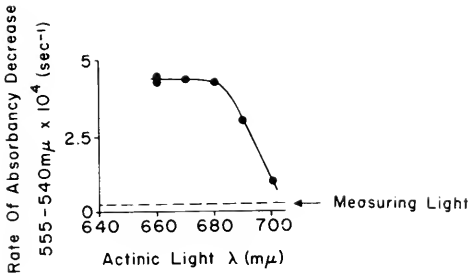

FIGURE 6.

Figure 5. Illustrating the effect of light of various wavelengths in the red region upon the rate of cytochrome f oxidation in the Swiss chard leaf at 77° K. Time proceeds from left to right. Measuring light slits set at 1 mμ cause little measurable oxidation. However, illumination with 680 mμ gives rise to a rate of 4×10^{-4} per second per leaf thickness (0.4 mm). The times at which the illumination is changed to various other wavelengths is indicated on the diagram. Experiment 63-15.

Figure 6. A plot of the effect of red illumination upon the rate of cytochrome oxidation for a Swiss chard leaf at 77° K. The points are taken from the initial velocity of a number of similar Swiss chard leaves. The rates of absorbancy change are per leaf thickness (0.4 mm). The diagram also indicates the rate obtained with measuring light only. The data are corrected for the small change of energy distribution of the monochromatic light over the spectral interval. Experiment 63.

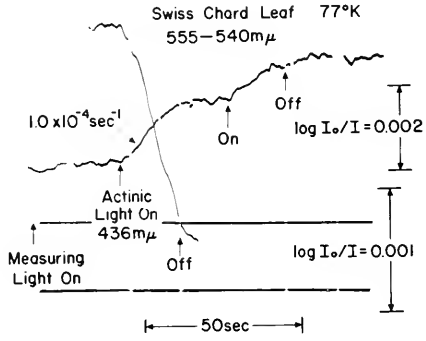

Figure 7. Activation of cytochrome f oxidation in Swiss chard leaf at 77° K with 436 mμ actinic light obtained from a medium pressure mercury arc (see Fig. 6). The convention used in recording is similar to that in previous experiments. Experiment 61-10.

thereby, a "difference spectrum" for the actinic effect. This is illustrated in Fig. 4 for Swiss chard leaves and for spinach leaves. In both cases there is a large diminution of absorption which has a maximum very near 555 mμ. In spinach leaves, there is a possibility that a satellite band characteristic of cytochrome f is observed, although further experimentation is desirable to ensure this. This satellite band is not observed in Swiss chard leaves, although insufficient data are available at present to substantiate this difference. It is significant, however, that the band at low temperature is at 555 mμ, whereas the peak in acetone extracted spinach quantasomes is clearly at 552 mμ (13). It is probable that this difference is a real one, that cytochrome exists in a different state in the leaf than in the acetone treated chloroplasts or in the extracted pigment.

Relative quantum efficiency for cytochrome f oxidation.

As the preceding Figures clearly indicated, the velocity of cytochrome f is under control of the intensity of the actinic light. It has occurred to us that it would be of considerable interest to determine the effect of the wavelength of light upon cytochrome f oxidation since we have here for the first time, the isolated primary chemical event of the leaf.

Instead of the fixed wavelength actinic beam a Bausch and Lomb 200 mm focus grating monochromator (1200 line grating) is employed, and is illuminated with a tungsten lamp. The energy distribution of this combination is found to be practically flat in the region of interest (620-710 mμ). Manual rotation of the wavelength knob controls the rate of this chlorophyll at low temperatures. The monochromator was set with a 2 mm slit width (6 mμ spectral interval) which proved to be adequate to give rates of oxidation of cytochrome f (see Fig. 5), large compared with the rate caused by the measuring light.

As illustrated by Fig. 5, a Swiss chard leaf cooled at 77° K illuminated first with a measuring light, it is seen that the rate caused by the measuring light is insignificant. When the 680 mμ light is turned on an abrupt deflection of the traces is observed which proceeds considerably more rapidly for 680 mμ than it does for 690 mμ. In fact, when 700 mμ light is employed, the trace is nearly horizontal. The deceleration is, however, reversible, and when 670 and then 680 mμ radiation is employed, there is an abrupt acceleration. Since the course of cytochrome f oxidation follows an approximately exponential function, measurements of the rate were restricted approximately to the first third of the course of the reaction.

Furthermore, relative rates on adjacent segments of the curve were employed.

A summary of the results of the number of experiments is plotted in Fig. 7. On the ordinates are represented the rates of absorbancy decrease at 550-540 mμ (as measured from the slope of the curve similar to that of the preceding Figure), the abcissa are values of wavelength of the actinic light. The dashed line at the bottom of the Figure is the rate obtained by the measurement light only, and this is seen to be negligible for all wavelengths employed. The graph shows a plateau in the region of 660-680 mμ with an abrupt decrease at 690 and 700 mμ. The curve may be interpreted as a decrease in quantum efficiency of electron transfer between chlorophyll and cytochrome f; the efficiency falls to half its value at approximately 695 mμ. A similar fall-off is found with frozen chloroplast suspensions. One measurement of a decrease of efficiency at 640 mμ reported in the oral presentation was found to be in error.

Absorbancy changes at 700-705 mμ

In order to observe the response of the 700 mμ pigment under conditions where the response of cytochrome f can also be measured, we have employed actinic excitation at 436 mμ from a medium pressure mercury arc. With this illumination, a rate of cytochrome f oxidation corresponding to 1.0×10^{-4} OD units/sec was obtained as is indicated in Fig. 7, where illumination occurs for a period of 20 seconds. A second period of 20 second illumination carries the reaction nearly to completion.

If the measuring wavelengths are now changed to those appropriate to the 700 mμ pigment (705-635 mμ) Fig. 8, we find a more rapid rate of change of absorbancy when the measuring light is on, and note a considerable increase of rate during the 20 second period of actinic illumination at 436 mμ. Since the sensitivity is lower than in the recording of cytochrome f, the rate corresponds to 2.7×10^{-4} OD units/sec. A second interval of illumination causes completion of the reaction.

A number of features of the reaction are of importance. First, both the reactions proceed when the measuring light is turned on, and are accelerated simultaneously when illuminated with actinic light. This point will be taken up in the Discussion.

By repeating the experiment of Fig. 8 with different leaves

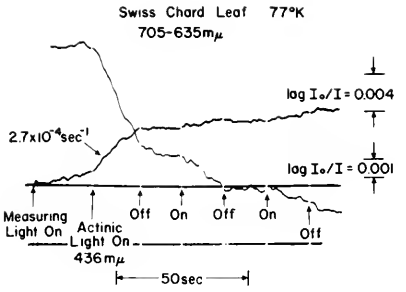

Figure 8. Measurements of thekinetics of absorbancy change in the region of "P_{703}" in a Swiss chard leaf frozen at the temperature of 77°K. In this case, the measuring light (slits 1 mμ) causes appreciable activation of the absorbancy change. However, the increase on illumination at 436 mμ is marked. The rate of absorbancy changes is calculated per leaf thickness (.4 mm). Experiment 61-10.

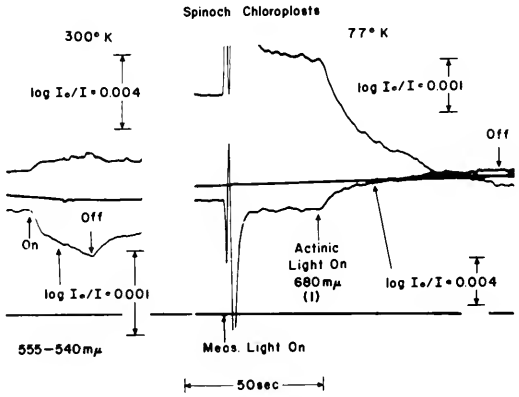

Figure 9. A comparison of room and low temperature response of cytochrome f in a suspension of spinach chloroplasts (279 μg chlorophyll a/ml). The actinic light employed in both cases is of the same intensity and a wavelength of 680 mμ. The appropriate absorbancy scales for the two traces are indicated. Upon illumination, the absorbancy at 555 mμ decreases with respect to that at 540 mμ. Experiment 57B 56, 57.

Comparison of Cytochrome f and P_{700} kinetics in Swiss Chard leaves at 77°K (Expt. 64)

Expt. No.	X	ΔOD_{max}	$\Delta OD \times 10^4$ (sec^{-1})	Assumed $\Delta\epsilon$ ($cm^{-1} \times M^{-1}$)	d (mm)	Δc μM	$\frac{\Delta C}{\Delta T}$ μM/sec	Chlorophyll μmoles/kg leaf wet weight
1	555-540	0.0060	1.0	20	0.4	.76	.12	400
8	705-635	0.0125	2.7	80	0.4	.38	.085	400

TABLE II

and with a variety of measuring wavelengths, the difference spectrum for the 700 mμ pigment can be determined, and it is found to lie between 700 and 705 mμ in accordance with the results of Witt (7) and Kok (14). At low temperatures, the half width of the band is roughly 10 mμ.

Data calculated from Fig. 8 are summarized in Table II. The maximum absorbancy change in the long wave region is about twice that at the shorter wave region and the rate in the longer wave region three times that in the shorter wave region. We make the usual assumption that the extinction coefficient of the 700 mμ compound is the same as that of chlorophyll a at 680 mμ, and further assume that the extinction coefficients of cytochrome f and P_{700} change in proportion at low temperatures. On this basis, the ratio of the concentrations of the two substances is about 2 to 1. The ratio of the rates is of more interest; the molar rates of absorbancy change are about equal (actually, the cytochrome f rate is 1.4 times the P_{700} rate). It should be emphasized, however, that these comparisons are only approximate and may be revised when more accurate data are obtained.

DISCUSSION

The observation of cytochrome f oxidation isolated from other reactions of the complex matrix of photochemistry and biochemistry allows a detailed study of the mechanism of the electron transfer reaction between cytochrome f and chlorophyll. While it is not the purpose of this preliminary note to discuss this in detail, it is apparent that the accurate recordings of the kinetics in the illuminated frozen leaf will be of great advantage in further experimentation.

The present discussion will be limited to a consideration of the low temperature oxidation of cytochrome f in relation to the oxidation of cytochrome c_2 in photosynthetic bacteria and to the absorbancy change at 700 mμ.

Properties of the low temperature oxidations of cytochrome f and cytochrome c_2.

Our previous observation of the kinetics of oxidation of cytochrome c_2 in Chromatium at liquid nitrogen temperatures (11) is supported by the observation of the cytochrome f kinetics in leaves. These results greatly extend those obtained on aged chloroplast suspensions by Witt (7). It is clear from these results

that the electron transfer reaction between cytochrome and chlorophyll is very unusual; its temperature insensitivity underlines the juxtaposition of these two metalloporphyrins in a way so that collision reactions are not required for electron transfer. In this paper, we have not yet determined the quantum requirement for cytochrome f oxidation at the low temperature, but it can be inferred from the kinetic data of Fig. 4 where the low temperature rate is considerably faster than the room temperature rate, that the quantum efficiency is probably high.

It is of interest that the oxidation of cytochrome f at low temperatures is rapid in aged spinach chloroplasts as found by Witt and extended by us to fresh spinach chloroplasts (Fig. 9). This is not true of Chromatium chromatophores which have been found by us (15), to be more temperature sensitive than the reactions in the intact cells; the reaction comes to a halt at 77° K. It has further been noted by Duysens (16) that some algae show a rate limitation in cytochrome oxidation at low temperatures. Whether or not this is due to a basic difference in the mechanism or whether the essential orientation of cytochrome and chlorophyll is deranged during the freezing of some materials and not others cannot be stated at the present time.

In preliminary experiments we have examined the effectiveness of red light in cytochrome f oxidation and find that the rate of oxidation falls to half the maximum value at approximately 695mμ. In other words, the quantum efficiency falls to half maximal val- at 695 mμ. This result gives evidence for the intimate identification of cytochrome f with system I in the leaf.

Kinetics of cytochrome f and P_{700}

The possibility of measuring absorbancy changes corresponding to these two components in the frozen leaf would appear to present optimal conditions for a critical evaluation of the possibilities of their interaction (14). Under these conditions, thermal reactions in which the two might be involved would be negligible and any possibilities for their direct interaction might be observable.

In the intact leaves, we routinely observe absorbancy changes at 555 and 705 mμ typified by the data of Table II, approximately double the absorbancy change at 705 as compared with 555 mμ. In fresh chloroplasts the absorbancy change at 555 mμ is observed in roughly the same relationship to the chlorophyll content as in the intact leaves (see Fig. 9). At 700 mμ, less than one fifth the absorbancy change is observed and this is partly reversible on cessation of actinic illumination. This result at 700 mμ may

be compared with that of Witt (3) who states that he was unable to observe any changes at 700 mμ in fresh chloroplasts. It is possible that the reversible light response at 700 mμ is associated with some damage to the leaves.

One possible explanation is based upon the idea that two light-induced oxidations occur at low temperatures. The disappearance of absorption at 700 mμ is concluded to represent an oxidation state of chlorophyll but its chemical configuration is quite unknown (14). The evidence for the oxidation of cytochrome f is firmly based upon the disappearance of the characteristic absorption band of ferrocytochrome f. It becomes, therefore, of considerable interest to determine which is oxidized more rapidly. As Table II indicates, the absorbancy decrease is relatively more rapid at 705 mμ than at 555 mμ. However, a comparison of Figs. 8 to 9 indicates that the reactions come to completion at about the same time. In Table II, we have attempted to make the comparison more meaningful by converting the rates of absorbancy change to molar rates, making assumptions which need, however, a detailed study and critical evaluation. However, the simple assumption that the extinction coefficient of cytochrome and chlorophyll bear the same relation to each other at low temperatures as they do at room temperatures brings the rates of the light-induced reactions of cytochrome f and P_{700} closely to the same range, cytochrome f being slightly faster than P_{700}. On the basis of any of a number of simple mechanisms, it appears that neither cytochrome f nor P_{700} is a rate limiting intermediate in the oxidation of the other.

Even qualitative aspects are useful in this respect. First, there is no induction period in the light-induced oxidation of either of these pigments which would suggest a sequential reaction, i.e., a delay in the oxidation of cytochrome f prior to the oxidation of P_{700} and vice versa. This lack of induction period is also observed at the measuring light and actinic light intensities. One mechanism, which appears to meet the needs of the kinetic data is that cytochrome f and P_{700} are interacting with different chlorophyll molecules, with cytochrome f being at the active center of the photosynthetic unit and P_{700} being at a chlorophyll molecule which is on the energy transfer pathway from the initial receptor to the active center. It is apparent that a very detailed examination of the quantum requirements for these two light-induced oxidations would be of great importance in this respect. Since both oxidation reactions occur simultaneously, their quantum requirements should be additive.

Actually there would appear to be no need for a spectroscopically distinct form of chlorophyll to be formed simultaneously with the oxidation of cytochrome f, presumably the metastable state of chlorophyll which acts as the energy trap for the initial light reaction could accept an electron from cytochrome and transfer it to the electron donor in a temperature insensitive reaction, without the need for the accumulation of a measurable amount of a chlorophyll intermediate.

A second mechanism that fits the needs of the experimental data and which takes into account the certainty with which the disappearance of the 555 mμ band of cytochrome f indicates an oxidation of ferro-cytochrome f is that the P_{700} absorption is actually that of a reduced chlorophyll intermediate. Under these conditions, an exact correspondence of the molar rates of change would be expected. Before this hypothesis can be considered seriously, the apparently sound basis upon which it has been concluded that P_{700} is an oxidized form of chlorophyll must be critically reexamined (14).

SUMMARY

1. The demonstration of the temperature insensitive oxidation of cytochrome f in leaves of three types of plants is reported; difference spectra are provided and the kinetics of the change are measured.

2. The quantum efficiency for cytochrome f oxidation falls rapidly in the red region, a half maximal efficiency is obtained at approximately 695 mμ.

3. The rates of light-induced absorbancy changes due to cytochrome f and to P_{700} have been compared at temperatures of liquid nitrogen. While the times for completion of the two reactions are approximately the same, the 700 mμ change corresponds to a larger absorbancy and hence, has a larger optical rate. However, conversion to a molar basis (assuming that the effect of temperature upon the extinction coefficient of chlorophyll is the same as that upon cytochrome f) brings the two rates approximately into agreement at the two values of light intensity employed here.

4. Two reaction mechanisms fit the experimental data: a) that cytochrome f and P_{700} react at different chlorophyll molecules, cytochrome f presumably reacting at the photosynthetically reactive center, and P_{700} acting at a chlorophyll molecule involved in energy transfer; and b) that cytochrome f and P_{700} react at the same active center and that cytochrome f is an electron donor and P_{700} an electron acceptor. The first mechanism is consistent with the data of other workers; the second is not in agreement with studies of the oxidation-reduction reactions of P_{700}.

FOOTNOTE

In the verbal presentation of this paper, it was noted that the absorbancy decrease at 705 mμ reverted towards the baseline after cessation of actinic illumination. The experiments have been repeated, but the results are irregular. Since the main conclusions on the relationship of cytochrome f and P_{700} do not depend upon this observation, this paper reports only observations under conditions in which the absorbancy change due to illumination at low temperatures is apparently stable.

ACKNOWLEDGMENTS

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LIGHT INDUCED OPTICAL CHANGES IN GREEN LEAVES

Walter Bonner and Robert Hill

INTRODUCTION

In a previous account of some work by the present authors (1) accumulated evidence was presented suggesting that light initiated oxidation-reduction reactions in higher plants involved cytochromes f and b_6. As yet, there are no unequivocal data relating to rapid changes in the oxidation states of cytochromes f and b_6 on illumination of green leaves or isolated chloroplasts. Light induced optical changes, which have been said to correspond to cytochromes, have been described in isolated chloroplasts of higher plants by Lundegårdh (2), James and Leach (3), and Müller, et. al.(4). The present paper describes the rapid oxidation of cytochrome f which occurs on illumination of green leaves and, in addition, some observations pertaining to cytochrome b_6. An abstract of some of this work has been published (5).

METHODS

Etiolated leaves of mung bean (Phaseolus areus) were obtained from seedlings grown in the dark at 25°. Spinach leaves were obtained from locally grown plants; spinach chloroplasts were prepared by standard procedures. Optical measurements were performed either with a double-beam differential spectrophotometer or with a rapid scanning split-beam spectrophotometer; both of these instruments were similar to ones already described (6). Leaves were mounted on a specially constructed rack which fitted into a moist chamber and suspensions of chloroplasts or algae were placed directly into the moist chamber. The basic design of the moist chamber has been described previously (7). Actinic light was provided with a Unitron Koehler illuminator equipped with suitable interference filters. In all experiments the photomultipler of the spectrophotometer was shielded from the actinic light with suitable filters. The visual optical observations were performed with a low-dispersion microspectroscope; the optical path of the comparison prism was equipped with two wedged troughs.

RESULTS

Cytochromes f and b_6 are easily observed in acetone extracted chloroplasts and in etiolated leaves (1). It is also apparent

that in certain selected algal mutants it is possible to make direct observations on the chloroplast cytochromes (8) and indeed in one of them, to show light induced oxidations of cytochrome f and of a b-type cytochrome (8,9). The small etiolated leaves of mung bean seedlings show intense bands of both plastid cytochromes when the leaves are observed through a direct vision microspectroscope. Furthermore, it is possible to record a spectrum of the two cytochromes in a suspension of plastids prepared from etiolated mung bean leaves. Figure 1 shows such a spectrum.

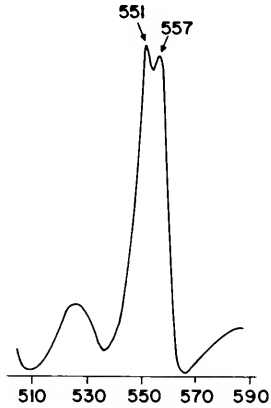

Fig. 1 Low temperature (77°K) difference spectrum, reduced-oxidized, of a suspension of plastids isolated from etiolated mung bean leaves.

The small etiolated mung bean leaves, minus their mid-ribs could be relatively easily layered on a special rack which fitted into the moist chamber and which in turn, fitted into the optical path of the double-beam differential spectrophotometer. Both of the authors are deeply indebted to Dr. Hiroshi Ikuma who possesses great skill in the delicate operation of mung bean leaf mounting.

No light induced optical changes could be observed in the etiolated leaves, in the region between 500 and 580 mμ, until the leaves had been allowed to produce some chlorophyll. Greening of the leaves was accomplished by illuminating them, in the moist chamber, with low intensity red light. The filter used for green-

ing had a wide band with 70% transmission between 680 and 1000 mμ. Figure 2 shows the development of a light-activated response following chlorophyll formation in the mung bean leaves, a response that corresponds to the oxidation of cytochrome f.

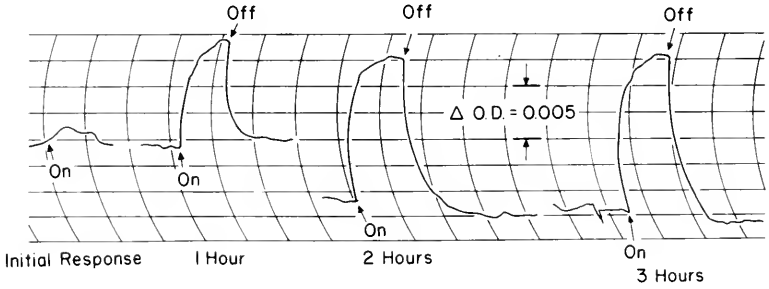

Fig. 2 Development of the light activated cytochrome f oxidation during greening of etiolated mung bean leaves. The times indicated refer to the number of hours the leaves were exposed to broad-band, low intensity red light.

Light induced cytochrome f oxidation could be observed in a relatively short time following initiation of chlorophyll formation. Maximal response was obtained after three hours of greening, at which time the leaves were very slightly tinged with green. Further greening of the leaves did not increase the extent of the light induced cytochrome f oxidation, but did markedly increase the rate of the response. A relatively high light intensity was required for cytochrome f oxidation in these partially greened leaves (Figure 3).

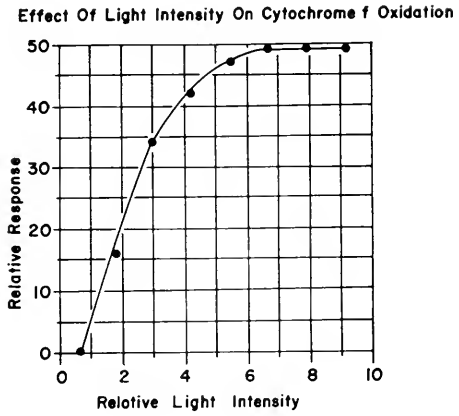

Fig. 3 The relation between extent of cytochrome f oxidation and light intensity in partially greened mung bean leaves.

Light induced cytochrome f oxidation was activated with 700-mμ actinic light only; 640, 660, and 680 mμ were ineffective. 700 mμ actinic light did not cause noticable greening of the leaves, a fact that clearly separates the events leading to the response and the activation of the response.

A spectrum of the 700 mμ light activated responses in partially greened mung bean leaves is shown in Figure 4, a spectrum that corresponds remarkably well to that of cytochrome f.

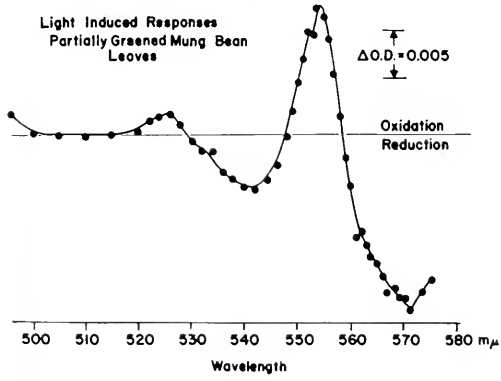

Fig. 4 The light activated optical responses of partially greened mung bean leaves plotted as a function of wave length.

The spectrum of Figure 4 shows the α-band of cytochrome f and some semblence of the β-band; the complete absence of the 518 response is worthy of note. In this spectrum 559 mμ appears to be isosbestic and at longer wave-lengths there is a region of decreased transmission, a decrease in transmission that could be interpreted as light induced cytochrome b_6 reduction. In these experiments there were suggestions that as the mung bean leaves formed chlorophyll the α-band of cytochrome b_6 became broader and shifted toward the red. However, if one measured, during greening, both the amount of chlorophyll and the amount of cytochrome b_6 using visual optical methods (a direct-vision microspectroscope, the optical path of the comparison prism being equipped with two wedged troughs containing respectively a standard chlorophyll a solution and a standard hemochromogen solution) it was observed that cytochrome b_6 gradually disappeared as the chlorophyll concentration increased but the α-band of cytochrome b_6 remained remarkedly sharp at 560 mμ as long as it could be observed. The results from experiments using both the differential spectrophotometer and the direct vision microspectroscope can be interpreted in three ways: 1) There is formation of a chlorophyll-cytochrome complex, a complex which could be similar to that of Takamiya et. al. (this symposium). In the case of the mung bean leaf the complex would be between cytochrome b_6 and chlorophyll a since only chlorophyll a is formed during these early stages of greening; 2) One is observing the light activated oxidation of cytochrome b_6, an oxidation that requires a higher chlorophyll concentration than the corresponding reaction with cytochrome f; 3) The fact that in the partially greened leaf (and in the fully greened leaf also) there is no light induced optical response in the region of cytochrome b_6 α-band absorption (560-570 mμ) while under the same conditions there is a rapid light induced oxidation of cytochrome f might point to the conclusion that cytochrome b_6 does not participate in light activated electron transport.

Oxidized cytochrome f, like oxidized cytochrome c, possesses a region of steadily increasing opacity in the region 560-570 mμ; the transmission decrease between 560 and 570 mμ shown in the spectrum of Figure 4 can be interpreted as being caused by the formation of oxidized cytochrome f. For this reason and because the spectrum of cytochrome b_6 remains sharp up to the point of its disappearance, the gradual disappearance of cytochrome b_6 with greening appears to be caused by a light activated oxidation of this component. In these experiments a light induced cytochrome b_6 reduction was observed, but only in ruptured chloro-

plasts. No light induced oxidations or reductions of cytochrome b_6 were found in whole chloroplasts or in green leaves.

Having learned the technique of observing light induced optical changes in partially greened leaves, it was relatively simple to record light induced cytochrome f oxidation in a large variety of fully greened leaves, in green algae (Chlorella, Chlamydomonas) and in blue-green algae. The light activated spectral responses in a fully green spinach leaf are shown in Figure 5 where again the characteristic assymetric α-band of cytochrome f is strikingly apparent, but unlike the partially greened mung bean leaf, the fully greened leaf shows the response at 518 mμ. There is no 518 mμ response in Euglena graciles or in Anacystis nidulans, an observation in confirmation of Olson and Smillie (this symposium) and of Amesz and Duysens (10).

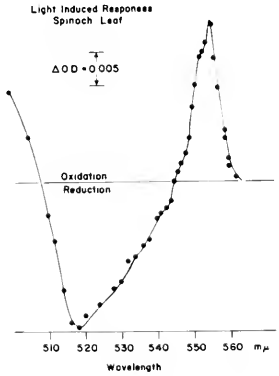

Fig. 5 The light activated optical responses of a fully green mature spinach leaf plotted as a function of wave length.

It is shown in another paper (Chance and Bonner, this symposium) that the light induced oxidation of cytochrome f proceeds at 77° K and the oxidation rate is more rapid at this temperature than at 25° C. Even so, the "on" and "off" responses of the partially greened bean leaves are remarkably fast at 25°C. The partially greened leaf has the terrific virtue that in the kinds of experiments described here there is a negligible effect of the measuring light on the cytochrome f response, a situation that depends on the low chlorophyll concentration and hence the high light intensity requirement and one that does not exist in fully greened tissues or cells. The "on" and "off" responses for

cytochrome f oxidation in partially greened mung bean leaves are shown in Figure 6. It may be seen in this figure that the dark reduction of cytochrome f is rapid, so rapid in fact that the techniques that have been used previously to observe light induced optical changes in chloroplasts (2,3) and in Chlorella (11) would fail in any attempt to observe specific light induced changes.

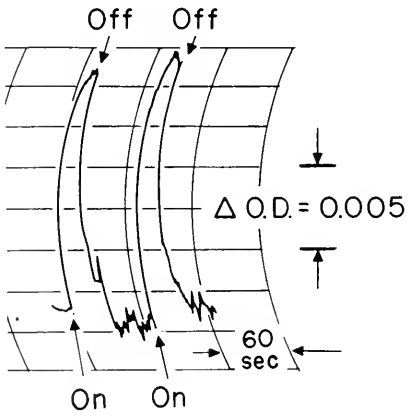

Fig. 6 An illustration of the time relations involved in light activated cytochrome f oxidation and the subsequent dark reduction of cytochrome f in partially greened mung bean leaves.

DISCUSSION

The paper documents, for the first time, specific light-induced optical changes in the partially greened leaf as well as in fully greened leaves and in green algae. This paper also confirms the observations of Amesz and Duysens (10) and of Olson and Smillie (this symposium) relating to the light-induced cytochrome f oxidation in Anacystis and in Euglena. The accumulated experience gained through comparison of the light-activated cytochrome f oxidation in partially greened leaves and in various fully greened leaves has emphasized the need for considerable care in investigations on fully greened leaves. Extra precautions are required because of a low light requirement that can be met, partially, by the measuring light. The partially greened leaf has three distinct advantages, compared to the green leaf: (a) better optical properties; (b) the high light intensity require-

ment for cytochrome f oxidation, a requirement that negates possible influences of the measuring light, and (c) the control of the development of light activated responses through control of the leaf chlorophyll content.

The observation that light-induced cytochrome f oxidation can be observed after an etiolated leaf has been allowed to form some chlorophyll is simply a prelude to the observations that now must follow. What is the role of cytochrome b_6, does it form a complex with chlorophyll, is it oxidized or reduced by light, or does it have any role in photosynthetic electron transport? Very precise data are now needed on rates of chlorophyll formation, development of the f response, development of oxygen evolution and carbon dioxide assimilation, plastoquinone formation, the time relations involved in the 518 response as well as correlations of the above lore with the light-induced completion of the chloroplast structure itself. Preliminary experiments have shown that many hours of greening are required before plastoquinone is formed. Because of the close association, in the minds of various investigators, between plastosquinone and the 518 response, it is very important to follow the development of this response during greening. It is always a pleasure, however, to find that the solution of a specific problem points immediately to other specific and more challenging problems.

SUMMARY

1. Cytochromes f and b_6 can be observed directly, by means of a microspectroscope, in intact leaves of dark grown mung bean seedlings. Spectra of these same cytochromes can be obtained from plastid suspensions prepared from such etiolated leaf tissue.

2. Following the development of a small amount of chlorophyll in the mung bean leaves, and while cytochrome b_6 is still visible in the microspectroscope, the absorption spectrum of cytochrome f can be plotted from the light activated optical responses as observed in a differential spectrophotometer.

3. Following more prolonged greening the α-band of cytochrome b_6, as observed visually, remains sharp but gradually disappears without noticeable change in the position of its maximum.

4. The absorption spectrum of cytochrome f can be plotted from the light activated optical responses, as observed in the differential spectrophotometer, in a variety of fully greened leaves and in green algae.

5. No light-induced optical responses corresponding to cyto-

chrome b_6 have been observed in either green leaves, whole chloroplasts derived from these leaves, or in algae. Light activated cytochrome b_6 reduction is found in ruptured chloroplasts.

ACKNOWLEDGEMENTS

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ACTION OF TWO-PIGMENT SYSTEM ON FLUORESCENCE YIELD OF CHLOROPHYLL *a*

W. L. Butler and N. I. Bishop

Light-induced fluorescence yield changes of chlorophyll *a* *in vivo* have been related to the two-pigment system of photosynthesis. Govindjee, et al.,[1] reported that the intensity of fluorescence emitted by chlorella, when illuminated with 670- and 700-nm light simultaneously, was less than the sum of the intensities obtained with the 670- and 700-nm beams separately. Butler[2] showed that the yield of chlorophyll *a* *in vivo* was reversibly increased by irradiation with red light and decreased by irradiation with far red. The action spectrum for the effect of far-red light in decreasing the fluorescence yield had a maximum at 705 nm[3] which was similar to the action spectra for the effects of long-wavelength light in enhancement phenomena of the second Emerson effect. Teale[4] reported that with green algae and chloroplasts the action spectrum for the effect of light in increasing the fluorescence yield had maxima at 470 and 650 nm and a shoulder at 670 nm, a typical action spectrum for the shorter wavelength pigment system in green plants. Duysens[5,6] also showed with red, blue-green and green algae that the fluorescence yield of chlorophyll *a* was increased by light absorbed by the shorter wavelength pigment system (which he called system 2) and decreased by the longer wavelength pigment system (system 1). We have also measured the action spectrum for the light-induced fluorescence yield increase in green leaves and algae and will report the results in the present paper.

The symbolism and theoretical framework introduced by Duysens[5,6] will be adopted in this paper. It will be assumed that pigment systems 1 and 2 both contained chlorophyll *a* and the accessory pigments (chlorophyll *b* in green plants and phycobilins in red and blue-green algae) but that most of the accessory pigment is associated with system 2. Also, according to Duysens, the chlorophyll *a* in system 1 is weakly or nonfluorescent, while the chlorophyll *a* in system 2 fluoresces with a variable yield that depends on the redox state of a quenching substance Q. A similar quenching substance was proposed by Kautsky, et al.[7] on the basis of a detailed kinetic analysis of fluorescence

transients following the onset of illumination. In the sympli-fied electron transport chain proposed by Duysens and Sweers[6]:

$H_2O \rightarrow$ (system 2) $\rightarrow Q \rightarrow$ cyt $\rightarrow P \rightarrow$ (system 1) $\rightarrow PN$

Q will quench the fluorescence of the chlorophyll a in system 2 but the reduced state, QH, will not quench. Light absorbed by system 2 will reduce Q to QH, thus increasing fluorescence while light absorbed by system 1 will oxidize QH to Q, thereby quenching the fluorescence.

P is a small amount of a chlorophyll absorbing near 700 nm. Kok[8] showed that this component, which he called P 700, is oxidized and thereby bleached by light absorbed by system 1 and that it is reformed by light absorbed by system 2. Butler[9] studied this component (denoted C-705 in his work) by low-temperature absorption and fluorescence excitation spectroscopy and showed that energy absorbed by a large bulk of chlorophyll a is transferred by inductive resonance to P. Presumably, P is the energy trap for system 1. Duysens and Sweers[6] have also proposed that Q is the energy trap for system 2. The chlorophyll transfers excitation energy to Q but not to QH. So far, there is no direct spectroscopic evidence for this energy trap. In the scheme proposed by Duysens, system 1 and system 2 should be removed from the path of electron flow. They drive the electron transport chain by energy transfer to P and Q.

Butler[3] attempted to account for the fluorescence yield changes of chlorophyll a on the basis of energy transfer from chlorophyll a to P but not to P_{ox}. This explanation, however, did not account for the action of far-red light in decreasing the fluorescence yield. Duysens' scheme, in which Q is the quencher of system-2 chlorophyll, accounts satisfactorily for the experimental observations on fluorescence yield changes and relates these to changes in the electron flow along the electron transport chain. The fluorescence yield measurements thus become a convenient assay to determine if the electron flow is functioning. Such measurements will be reported in the present paper on mutants of Scendesmus which have specific blocks in the photosynthetic electron transport chain.

METHODS AND MATERIALS

The instrument, previously[3] used to measure the relative fluorescence yield immediately following a brief actinic irradiation, was modified so that the fluorescence excited at low intensity could be measured during the actinic irradiation. The

modified instrument was similar in principle to that used by Duysens (5). The fluorescence was excited by a weak (50 ergs/cm^2/sec), 650-nm monochromatic beam which was chopped at 360 cycles/sec. The sample could be simultaneously illuminated with a high intensity (1000 to 2000 ergs/cm^2/sec), monochromatic actinic beam. A cut-off filter, placed between the sample and phototube, blocked the low-intensity, measuring beam and the high-intensity, actinic beam but transmitted the fluorescence of wavelengths longer than 710 nm. It was previously shown with a green leaf (3) that the light-induced fluorescence changes, even when limited to wavelengths longer than 730 nm, were due to changes in the yield of chlorophyll a fluorescence. The photometer incorporated a tuned amplifier which was tuned to the chopping frequency of the measuring beam so that the alternating fluorescence excited by the chopped, low-intensity beam was measured but the constant fluorescence excited by the steady, high-intensity, actinic beam was not. The actinic beam changed the fluorescence yield which resulted in a change in the intensity of fluorescence excited by the chopped beam. The intensity of fluorescence excited by the actinic beam was monitored with a D.C. voltmeter which measured the drop across the anode resistor. The intensity of the actinic light was adjusted at each wavelength such that the intensity of fluorescence excited by this beam was about 20-fold greater than that excited by the measuring beam.

Absorption spectra were measured at -196°C with a single-beam recording spectrophotometer similar to one described previously (10). The spectrophotometer could also record derivative spectra by differentiating the signal from the photometer electrically.

Measurements were made on green bean leaves, on spinach chloroplasts and on suspensions of Scenedesmus. The Scenedesmus were wild-type cells and two classes of mutants which have been described previously by Bishop (11,12). One class of mutants ("CO_2" mutants) will not fix CO_2 in the light but will evolve O_2 in a quinone Hill reaction. The other class of mutants ("O_2" mutants) will not evolve O_2 but will photoreduce CO_2 in a hydrogen atmosphere.

RESULTS AND DISCUSSION

Action spectra for the effects of light on the fluorescence yield of a green leaf in air and in nitrogen are shown in Fig. 1. The fluorescence yield of the leaf is somewhat greater in nitrogen presumably because Q is more reduced. The horizontal lines show the relative intensity of fluorescence in the absence of

actinic light. In the presence of monochromatic actinic light, the intensity of fluorescence excited by the weak, chopped, 650-nm beam is given by the curve through the points. The intensity of actinic light was adjusted at each wavelength to give the same level of fluorescence as measured by the D.C. voltmeter. This procedure was adopted to make sure that approximately the same energy was absorbed at each wavelength. The intensity of actinic light at 550 nm was 50 percent greater than that at 650 nm because less light was absorbed at 550 nm. This method does not insure that precisely equal energy will be absorbed at all actinic wavelengths because the fluorescence yield appears to vary approximately 20 percent with wavelength across the visible spectrum. Adjusting to a constant level of fluorescence is more valid, however, than using a constant intensity of actinic light which would make the red and blue maxima in Fig. 1 more pronounced simply because those wavelengths were absorbed more

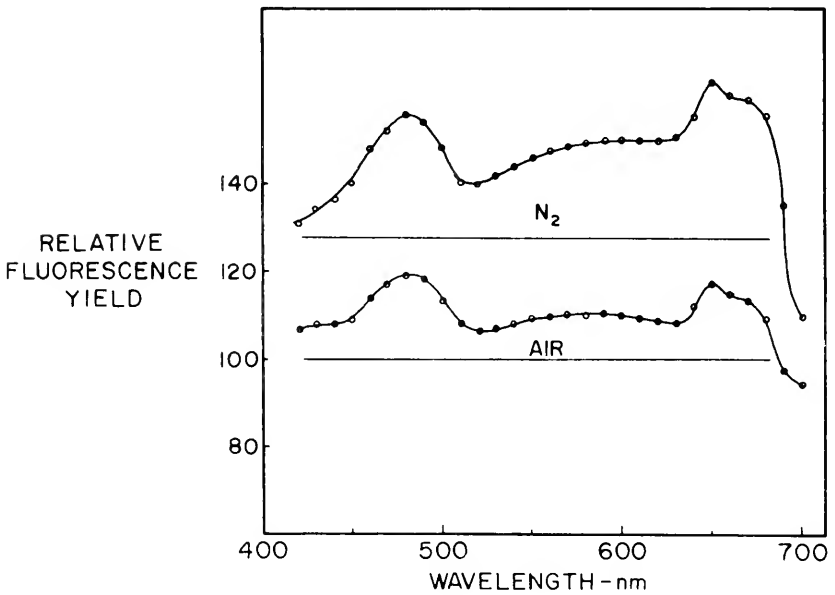

Fig. 1. Relative fluorescence yield of green bean leaf in the presence of actinic light vs. wavelength of actinic light. Details of measurement in text.

effectively. The action spectra of Fig. 1 are strickingly similar to the enhancement spectrum for chlorella shown by Myers and Graham (13).

The action spectra show that the wavelengths absorbed preferentially by system 2 are the most effective in increasing the fluorescence yield. Green plants do not show a clear-cut response to system-1 absorption because of the spectral overlap between systems 1 and 2. Beyond 680 nm, however, the absorption spectrum of system 2 falls markedly and the direct absorption by P becomes more important so that Q can be driven largely to the oxidized state by far-red light. It was previously shown that the most effective wavelengths for decreasing the fluorescence yield are those absorbed directly by P (3). Far-red light does not depress the fluorescence yield of the leaf in air much below the value for a dark leaf. Thus, Q is largely oxidized in the dark in air.

The fluorescence yield which obtains when the Q is largely reduced can be observed by adding DCMU. This herbicide apparently blocks the electron transport chain between Q and cytochrome so that P cannot oxidize QH (6). The addition of DCMU causes the fluorescence yield to increase approximately 3-fold. This maximal fluorescence yield also obtains momentarily during the transient fluorescence burst which occurs when a darkened leaf is first placed in bright light. The effect of DCMU on spinach chloroplasts is shown in Fig. 2. The chloroplast preparation without DCMU shows a typical two-pigment response. Light at 650 nm increases the fluorescence yield somewhat more than 600-nm light and much more than 690-nm light. After the addition of DCMU (10^{-5}M) the fluorescence yield increased slowly under the influence of the weak, measuring light and abruptly with the 650-nm actinic beam. The fluorescence yield drops somewhat when the actinic light is turned off, indicating that QH can be oxidized to some extent either by a back reaction or by dark metabolism. The light-induced fluorescence yield in the DCMU-treated chloroplasts is independent of wavelength, showing that system 1 or P have no influence on Q. The small drop at 690 nm is an artifact due to the small amount of the 690-nm actinic light which leaks through the 710-nm cut-off filter. In the case of the chloroplasts without DCMU, the marked depression of the fluorescence yield at 690 nm is due largely to the oxidation of QH by system 1 and P.

The light-induced fluorescence yield changes have been used to determine if both pigment systems were operating in mutants

of Scenedesmus. The fluorescence measurements on the wild-type Scenedesmus cells in Fig. 3 show the typical two-pigment control: The fluorescence yield is greater in 650-nm light than in 600-nm light and is markedly depressed by 690-nm light.

One set of mutants, called "CO_2" mutants, fails to fix CO_2 in the light but will evolve O_2 in a Hill reaction. The fluorescence measurements in Fig. 3 show that the fluorescence yield of the "CO_2" mutant is about 3-fold greater than that of the

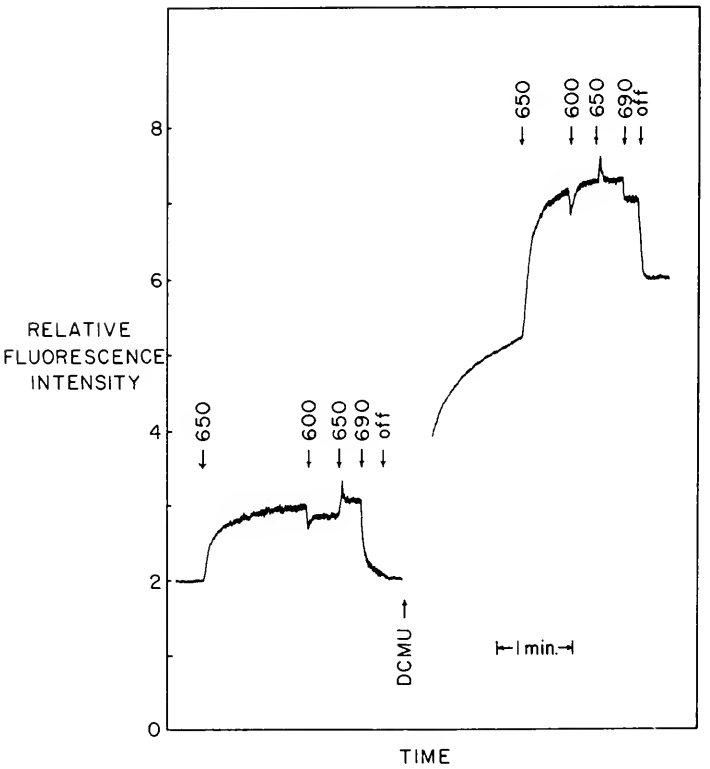

Fig. 2. Relative intensity of fluorescence (λ_F < 710 nm) from spinach chloroplasts excited by low-intensity, chopped, 650-nm beam in presence and absence of constant, monochromatic, actinic beam of wavelength indicated. Effect of 10^{-5}M DCMU shown.

wild-type cells. The fluorescence yield is increased further in the presence of actinic light, but all wavelengths are equally effective. The same results were obtained by adding DCMU to the chloroplasts in Fig. 2 and the same results would have been obtained by adding DCMU to the wild-type Scenedesmus cells. The fluorescence measurements indicate that system 2 reduces Q in the light but that the electron transport chain is blocked someplace between P and Q because system 1 and P cannot oxidize QH. The block in the electron transport chain prevents the reduction of TPN which is needed for CO_2 fixation but does not prevent system-2 activated transport of electrons from H_2O through Q to benzoquinone.

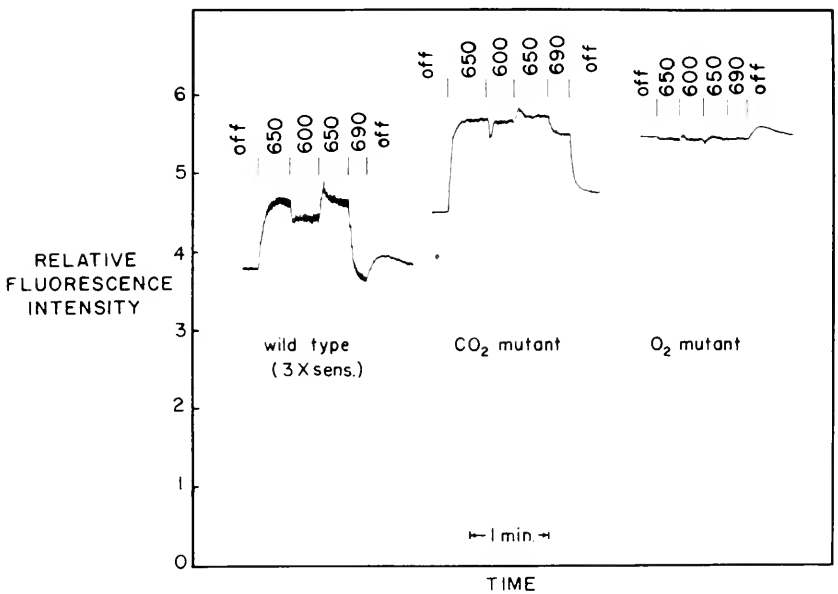

Fig. 3. Relative fluorescence intensity (same measurement as Fig. 2) from Scenedesmus wild-type, "CO_2" mutant No. 8 and "O_2" mutant No. 11 cells. Sensitivity of fluorescence measurement was increased 3-fold for wild-type cells so that the fluorescence yield of the wild-type cells is approximately 1/3 that of the mutant.

The other set of mutants, called "O_2" mutants, fails to evolve O_2 in the light but will photoreduce CO_2 in a hydrogen atmosphere. These mutants show a high fluorescence yield (Fig. 3) which is not affected by actinic light. These measurements are consistent with the photochemical activities of the "O_2" mutants which indicate that system 1 is operating, since CO_2 can be photoreduced with H_2, but that system 2 or some reaction closely allied with O_2 evolution is not functioning.

Low-temperature absorption and derivative spectra of the wild-type and mutant cells are shown in Fig. 4. The derivative spectra were used primarily to distinguish the presence of P, the small amount of chlorophyll absorbing near 700 nm. The

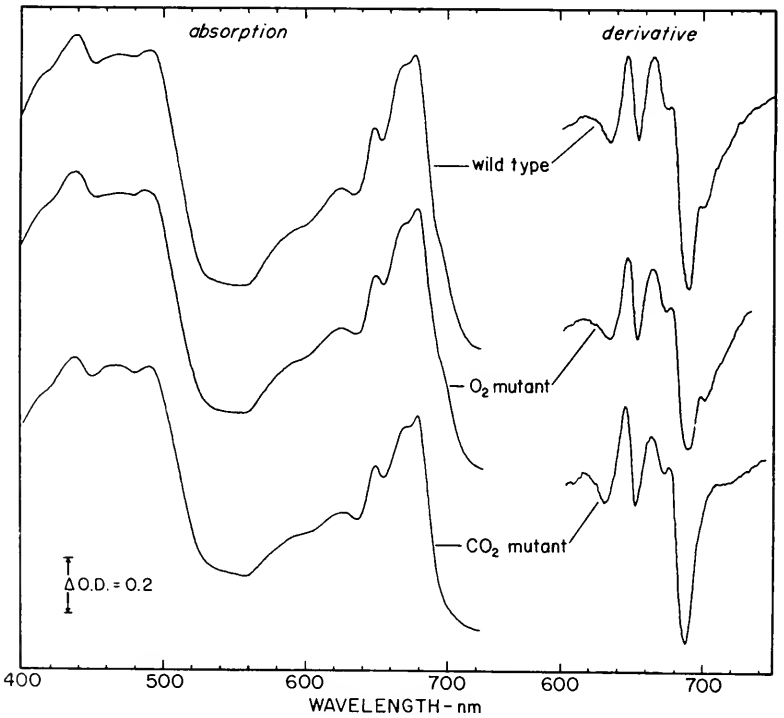

Fig. 4. Absorption and derivative spectra of Scenedesmus wild-type, "O_2" mutant No. 11 and "CO_2" mutant No. 8 cells at -196°C.

absorption spectra show that essentially the same amounts of chlorophyll b, chlorophyll a-670 and chlorophyll a-680 are present in all samples. Thus, the mutants carry a normal compliment of pigments in systems 1 and 2. P is present in the wild-type cells and in the "O_2" mutant, as shown by the slight shoulder in the absorption spectra on the long-wavelength side of the main chlorophyll-absorption band and by the maximum in the derivative spectra near 700 nm. The spectra for the "CO_2" mutant, however, do not show the presence of P. Thus, in these mutants, system-1 pigments cannot affect the redox state of Q because of the block in the electron transport chain at P. System-2 pigments, however, can cause Q to be more reduced in the light. The high fluorescence yield of these mutants in the absence of actinic light suggests that the Q is largely reduced in the dark. It would be of interest to determine if the cytochrome in these mutants could be reduced in the light by system 2 and oxidized in the dark as is Q.

The block in the "O_2" mutants has not been localized. The absence of a light affect on the fluorescence yield shows that Q is not being affected by light even though the absorption spectrum indicates that system-2 pigments are present. The high fluorescence yield suggests either that Q is fully reduced in the dark or that it is not present. The absence of Q in the "O_2" mutants would be analogous to the absence of P in the "CO_2" mutants.

The fluorescence yield changes are consistent with the photosynthetic electron transport chain and with the photochemical activities of the wild-type and mutant cells. These measurements provide a rapid and convenient method to study metabolic inhibitors and mutations which affect photosynthesis.

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PRINCIPLES OF A THEORY OF ENERGY UTILIZATION IN PHOTOSYNTHESIS

James Franck and J. L. Rosenberg

The present paper is an abstract insofar as it leaves out many details for the sake of brevity. An extended discussion will be published elsewhere. A short introduction which will be neither historical nor comprehensive in literature citations, will be presented first.

Our theory developed slowly from early attempts to find a general point of view for the meagre evidence available at that time. They were mostly based on van Niel's principle of water splitting, certain kinetic phenomena, and fluorescence observations. Along with proposals for which a sound experimental basis existed were a number of guesses which, under the influence of subsequent experimentation, became obsolete. One of the deductions which we regarded, and still regard, as well founded is that in green plants the excitation energy collected by the bulk of the plant pigments is transferred always to one center of photochemical activity, namely a chlorophyll a molecule in a special position which enables it to use the energy for the photochemical reactions of photosynthesis. Although this statement referred originally only to photosynthetic units in green plants, we extended it later to those in all plant cells. The energy-collecting part of the non-photochemically active regions of the unit contains the "protected" pigments, so-called because of the lack of contact between these dye molecules with water and its solutes. Their nature of the pigments may vary in different classes of plants or even from unit to unit within a given plant cell. In green plants, where all units contain chlorophyll a chlorophyll b, and β-carotene, the energy transfer to the lowest available excitation level is so quick, in accordance with well-known principles applied to dense and partly ordered systems, that the energy absorbed by all dyes reaches the exposed site as chlorophyll a excitation. The excitation arriving at the reaction center is usually that of the first excited singlet state, with the consequence that fluorescence visible during photosynthesis at non-excessive light intensities is predominantly the red fluorescence of chlorophyll a.

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Actually chlorophyll exists in the units in two different modifications, as the occurrence of a minor red-shifted set of absorption bands indicates. One of these modifications is usually present to a small extent and is contained in only a few units, so that it can exert only a minor influence on photosynthesis. (At this point we must state that in our previous publications we ascribed the weak spectrum to a so-called n-π transition which would be present in all units. We used special assumptions to explain why the energy would not always flow to this level. Our reasons for abandoning this interpretation will be presented in the forthcoming extended discussion). Under these conditions intensity measurements of the red fluorescence during and in the absence of photosynthesis are an unequivocal criterion of the utilization of the arriving singlet excitation energy for photochemistry. If only singlet energy would be used with optimal quantum yield there should be no fluorescence during photosynthesis. If, on the other hand, photochemistry always occurred by the action of metastable states formed from the excited singlet state in competition with fluorescence emission, the intensity of fluorescence would be independent of photochemical utilization. Observations showed that under the conditions mentioned above the ratio of the intensity of fluorescence in the presence or absence of photochemical utilization of excitation turned out to be very nearly one-half. This result was the same for three different methods of suppressing and permitting optimal photosynthetic activity. The obvious conclusion is that singlet and metastable excitation energy are used equally often for photosynthesis. That both singlet and triplet states are used for photosynthesis equally often is an indication that two photochemical reactions, one at the cost of each of these two states, must be coupled in such a way that they automatically occur at the same rate. That this coupling can be explained without contradicting any of the new important observations of the second Emerson effect and of the occurrence, spectroscopy, and photochemistry of the chlorophyll modification absorbing at 700 mμ, together with earlier and more recent studies of light emission of chlorophyll a is the theme of this paper. No attempt has been published so far to explain the emission and absorption phenomena of chlorophyll a together with the Emerson effect and reversible bleaching on the basis of a two-center model. We have attempted to adapt numerous published proposals to the above criteria but have not succeeded.

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NATURE OF THE TWO PHOTOCHEMICAL ACTS

For the application of a picture in which singlet and metastable state energy of chlorophyll a are used for the two photochemical steps of photosynthesis, we have to be more specific about these steps. From the work of Hill[1] and Kamen[2] it is known that cytochrome f (designated as Cyt_{red} and Cyt_{ox} for the ferro- and ferri-forms, respectively) plays a role in the photochemistry. If the light reactions consist of water-splitting, the cytochrome is regarded in our scheme as the primary OH acceptor while the H acceptor designated as A, is the primary photosynthetic oxidant. From the determination of the standard electrode potential of Cyt in the chlorophyll-cytochrome complex (Chl-Cyt) by Kok[3], we know that the Cyt_{ox} cannot evolve oxygen by dark reactions alone. Therefore a second photochemical step is needed on the Oh side by which Cyt_{ox} is reduced and another enzyme Y is oxidized to a state capable of yielding oxygen in subsequent dark reactions. Both Cyt and Y must be complexed with the exposed chlorophyll. The scheme is summarized in the following set of essential chemical reactions, where (1) and (2) are reactions of photo-excited Chl molecules, the first in the metastable state and the second in the excited singlet state, (1a) and (2a) represent sequences of enzymatic dark reactions.

$$A + (Chl^{*}_{met}\text{-}Cyt_{red}) \rightarrow AH + (Chl\text{-}Cyt_{ox}) \qquad (1)$$

$$2\,AH \rightarrow AH_2 + A \qquad (1a)$$

$$Y_{red} + (Chl^{*}_{sing}\text{-}Cyt_{ox}) \rightarrow (Chl\text{-}Cyt_{red}) + Y_{ox} \qquad (2)$$

$$Y_{ox} \rightarrow Y_{red} + \tfrac{1}{4}\,O_2 \qquad (2a)$$

GENERAL CONSEQUENCES OF THE SCHEME

The phenomena described in this section will be first applied to green plants, with omission of the long wavelength absorption (above 680 mμ). The consequences of the far-red absorption will be considered later.

Since the exposed chlorophyll comes in contact with many kinds of diffusible substances, a possible competition may exist for the

use of metastable excitation of Chl between A and other oxidants, some as harmful as molecular oxygen. Although in vitro experiments show that photo-oxidation of chlorophyll is much less likely than simple oxygen quenching of excitation, the former process does occur with a small yield. In any event, mere impacts of oxygen with Chl* may divert the use of excitation from photosynthesis. Since it is known that oxygen has a negligible influence on photosynthetic rates in regions of low irradiation intensity, we condlude that A must be present in at least a high enough concentration (10^{-4} molar or more) to win out over O_2 for the excitation. Among substances which do not meet this criterion for A are most of the materials present in catalytic amounts, including TPN. Phosphoglyceric acid, PGA, does meet the requirement and has been tentatively identified by us with substance A. In support of this view is the fact that other respiratory intermediates, normally present in smaller concentrations than PGA, become the substitute oxidants for Reaction (1) during periods of unavailability of PGA, such as cyanide inhibition, induction periods, and periods of greening of etiolated leaves[4,5].

At intensities approaching saturation the photosynthetic oxidant becomes limiting, and oxygen becomes a more effective competitor. This is the explanation for the strong depressing influence of oxygen on the saturation rate of photosynthesis. Although photo-oxidation of the exposed chlorophyll still proceeds with only a small quantum yield under these conditions, in the absence of a recovery process there would be a progressive accumulation of units whose exposed chlorophylls, oxidatively bleached, are unable to allow excitation to move to the reaction centers. Another competing process occurring with a probability rising with irradiation intensities is the collection by Chl^*_{met} of a second quantum of excitation before Reaction (1) has been sensitized. This double excitation, leading to photoionization and the associated afterglow, will not be discussed further in this paper, except for the remark that the connected bleaching would result in a disruption of the flow and utilization of excitation energy collected by the protected chlorophylls.

The disruption of energy flow may be avoided by a built-in recovery process, (3), analogous to the reaction described by Kok[3] and by Witt[6] for the special case of the 700 mμ absorbing form of chlorophyll. This recovery of normal exposed Chl would be rapid if the Cyt in its reaction center complex is

$$Chl^+ + Cyt_{red} \rightarrow Chl + Cyt_{ox} \qquad (3)$$

in the reduced form. The rate would then be so great that the reversible bleaching becomes non-observable in difference spectra experiments. Since photo-oxidation might attack any exposed Chl at random, efficient statistical recovery requires that practically all the Cyt molecules at the reaction centers be in the reduced form during steady-state illumination.

We now face the problem: how can most of the Cyt be in the reduced form? Simple kinetic analysis of our scheme shows that if each photosynthetic unit were functionally independent, half of all the Cyt should be in the reduced form and half oxidized during steady light-limiting illumination. In fact in the introduction we implied an independence of the units. We now qualify this statement by introducing an interaction at the level of the exposed chlorophylls. We postulate an energy migration of singlet excitation among the exposed chlorophylls in a larger superunit of the chloroplast. This proposal does not invalidate our conclusion that the far-red absorbing modification in green plants does not act as a trap for normal chlorophyll a singlet excitation, as will be discussed in the section on the Emerson effect. With this limited type of super-organization we gain the advantage that the steady-state fraction of Cyt in the oxidized condition is lowered to 1/2 n, where n is the number of participating units in the exchange. We visualize n to be of the order of magnitude of 100 or more. On this basis we can understand why good photosynthesis does not change the level of oxidized cytochrome, as shown by difference spectra.

That our scheme provides an automatic adjustment of equal opportunity for photochemical use of singlet and metastable excitations is obvious.

Next we must mention several consequences of our scheme for the fluorescence yield of exposed chlorophylls.

a) Butler has shown that freshly formed chlorophyll in a greening leaf has a fluorescence yield practically as high as that of chlorophyll in organic solvents.(7) Absolute measurements show that the fluorescence yield in vitro is about 25% while that of fully developed chloroplasts, in which only the exposed chlorophyll a is responsible for fluorescence, is 6% in the absence of photosynthesis. This difference may be ascribed to the influence of the heavy atom, the iron of the complexed Cyt, on the transition to the metastable state.

b) The fluorescence of the protected chlorophyll, if the absorbed energy is forced to remain inside the unit, should thus also be higher than of the exposed chlorophyll. If, for instance by photo-oxidation of the exposed chlorophylls during strong illumination or by other methods, the energy is prevented from migrating outward in the unit, fluorescence would be observed from the protected pigment molecules with up to a four-fold increase in yield. Indeed such observations have been made with a variety of external treatments[8,9] some of them destructive for the chloroplasts[10].

c) Our scheme forces us to the conclusion that half of the excited exposed chlorophyll molecules emit their full share of fluorescence, independent of the level or even absence of photosynthesis, while the other half, involved in Step (2), use singlet excitation immediately during good photosynthesis without any fluorescence. The consequence, that the lifetime of excitation of those emitting the fluorescence, is independent of photosynthesis, has been proved by several types of observation[10,11].

EMERSON EFFECT AND REVERSIBLE BLEACHING AT 700 mμ

Green Plants

We first discuss our interpretation of the far-red absorptions. After being forced by a number of new observations (to be described in the forthcoming extensive discussion) to abandon our previous idea that $n\pi$ transitions were responsible for the long wavelength absorption. We based our picture on a proposal originally made by Brody that chlorophyll exists within the photosynthetic apparatus partly in monomeric form and partly in aggregates[12]. This idea received strong support from the discovery by Olson et al. that the absorption at 700 mμ and the fluorescence above 700 mμ are highly polarized in the planes of the lamellae, while the major components observed in both absorption and luminescence are not polarized.[13,14] Specifically, we visualize most of the pigment molecules as being in a partly disordered, amorphous state within their units, while in some small fraction of the units there are regions of two-dimensional crystallinity bordering on the reaction center. As are all absorption peaks of the crystalline patches the red absorption is shifted slightly toward the inra-red and is split into two components. This is indeed the type of behavior observed by chlorophyll microcrystallites in vitro by Rabinowitch et al.[15] Fig. 1 shows the red shift and splitting

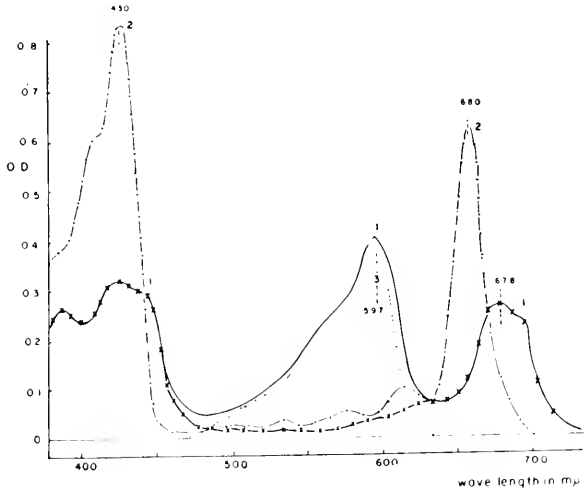

Fig. 1. Absorption spectrum of chlorophyll a in ether 10^{-5} M, curve 2; of colloidal chlorophyll suspension in 30% aqueous methanol, crosses of curve 1. Curves from reference (15).

of the long wave-length peak in their "colloidal" samples in comparison with monomeric material. Of the three chlorophyll a components found by French and Brown(16), we ascribe the main peak at about 670 to the amorphous chlorophyll, and the two minor peaks at about 680 and 700 mμ to the two differently polarized Davydov components of the crystalline chlorophyll. We note that the relative proportions of crystalline to non-crystalline chlorophyll may vary with culturing conditions and need have no particular stoichiometric value. Also, the extent of the absorption shift depends on the size of the microcrystallites(17) and a distribution of crystal size would be expected to cause broadening of the bands.

If our scheme makes possible a self-consistent explanation of the Emerson effect, it must provide the answer to five questions.

a) Why does the photosynthetic quantum yield fall in the far-red with increasing wave-length of absorption in the crystalline regions?

b) Why does the stronger Davydov absorption at 680 mμ give a good quantum yield while at the 700 mμ component the quantum yield is reduced to one-fourth or less?

c) Why can simultaneous or almost simultaneous absorption by the amorphous chlorophyll enhance the effectiveness of absorption by the crystals?

d) What is the role of the reversible photo- or oxidative bleaching and why is this bleaching observed mainly in the 700 mμ absorption?

e) In view of the energy migration through the super-unit which we introduced in the previous section, what prevents the degradation of excitation in the amorphous layers to the lowest excitation level of the crystalline layers, 700 mμ, by migration through the exposed chlorophylls?

To answer the first three questions we use the idea previously advanced by us that excitation provided by 700 mμ absorption is not sufficient to excite the singlet level of the exposed chlorophyll, which is an amorphous type molecule having its absorption at about 675 mμ whether it is attached to an all-amorphous unit or to a unit containing a crystalline patch. As a result excitation moving quickly in the crystal to the contact point with the exposed chlorophyll will suffer there a transition into a metastable state. A re-interpretation of Becker's observations(18) shows that the order of the metastable levels of crystalline and amorphous chlorophyll is the reverse of that of the singlet levels, and that a transfer of metastable energy to the amorphous exposed molecule will occur. The higher energy Davydov crystal component, 680 mμ is energetically so close to the singlet level of the exposed chlorophyll that by thermal fluctuation the 680 excitation may easily excite the singlet level of the exposed chlorophyll. In competition with this process, degradation of the 680 to 700 or even further to the metastable level is an inherently slower event.

If a reason can be found why the metastable state of an exposed chlorophyll is inefficient in sensitizing Reaction (2), we could understand the long wave-length decline and the enhancement. We considered several possible mechanisms, as follows:

a) Back-reaction between AH and Cyt_{ox}

As opposed to singlet excitation, metastable excitation cannot participate in the migration at the super-unit level. As a result, each Cyt_{ox} formed during a period of irradiation with 700 mμ only must wait a much longer period to receive a quantum of excitation for sensitizing Step (2). The Cyt_{ox} will thus have a greater chance to be destroyed by a competing dark reaction, such as one with AH. Our objection to this possibility is that it has no connection with the bleaching of crystalline chlorophyll, which plays such a great role in observed difference spectra.

b) Photo-oxidation of exposed chlorophyll.

Although photo-oxidation may occur with small probability with any type of irradiation, there are two important differences when the oxidized chlorophyll is at the reaction center of a crystal-containing unit. The first is that the resulting Chl^+ captures an electron from a neighboring crystalline chlorophyll because the ionization potential for the latter is lower. This in itself would slow the recovery reaction (3). The second difference is that half of the Cyt is oxidized at the steady state and is unavailable for the recovery reaction (3). This follows from the non-migration of metastable energy among exposed molecules and is in agreement with the observed increase in oxidized cytochrome during long wave-length irradiation. A progressive accumulation of units containing Chl^+ within the layers would lead to low efficiency if the trapped hole impedes the flow of excitation energy outward. Our objection to this possibility is that it is in contradiction to the finding by Gorindjee and Rabinowitch of the long wave-length decline and enhancement for the Hill reaction in the absence of oxygen[19].

c) Alternative electron transfer in competition with Step (2). We propose that triplet excitation can be used to sensitize Reaction (4), which is a reversal of the recovery reaction (3),

$$Cyt_{ox} + Chl^*_{met} \rightarrow Cyt + Chl^+ \qquad (4)$$

while singlet excitation cannot be so used. In the first instance, such an event would result in the detour of two quanta from photosynthetic purposes. Secondarily, it would produce much greater losses because the resultant bleached Chl would transfer its oxidation to crystalline chlorophyll. As in (b) above, the trapped Chl^+ within the layer would impede the outward flow of excitation until it has undergone recovery by a dark recapture of an electron from the Cyt_{red}. The basic idea

underlying this possibility is not so much an ad hoc hypothesis as it might seem. It is not unplausible to assume that in an excited Chl-Cyt complex more charge would tend to be transferred from the Chl to the Cyt for triplet excitation than for singlet under the influence of the mutual repulsion of two electrons with parallel spins. This explanation provides an interpretation of the Emerson effect, the enhancement by short-wave irradiation, and the 700 bleaching.

Finally, we have to discuss the possible role of the 700 mμ levels as a sink for shorter wave-length excitation. In our model, singlet excitation of amorphous chlorophyll molecules within a crystal-containing unit may be degraded to the 700 level during its passage through the crystal on the way to the reaction center. The converse is not true, however. Singlet excitation of exposed chlorophylls migrating through the super-unit might sensitize a crystalline molecule bordering on some reaction center, but would do so by preferentially sensitizing the stronger 680 level rather than the 700, with which the resonance overlap is poorer. As discussed previously, 680 excitation near the reaction center can be used with high probability for recreating singlet excitation at the exposed site. Thus, an overall degradation of singlet to triplet excitation at the super-unit level can occur with only a small probability.

Red and Blue-Green Algae

These algae can be incorporated into our general scheme if we postulate the following picture of the photosynthetic apparatus. Most of the chlorophyll is contained in crystal-containing units, so that even 670 mμ absorption has the effect of sending mostly triplet excitation to the reaction centers. The phycobilins are contained in units devoid of chlorophyll, but these units still have chlorophyll a as the exposed pigment at the reaction center. These phycobilinous units can provide interaction with the chlorophyllous units by way of super-unit energy migration in such a manner as to divide the photochemical chores in part between the two kinds of unit. Absorption in the chlorophyll units will be used predominantly for Step (1) while absorption in the phycobilin units will be used more than half the time in sensitizing Step (2).

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THE MECHANISM OF PHOTOSYNTHESIS

Eugene Rabinowitch

1.

Photosynthesis is a tripartite process, as indicated schematically in figure 1--one reaction set dealing with the conversion of CO_2 to organic matter, one with the liberation of oxygen from water, and one with the conversion of light into chemical energy.

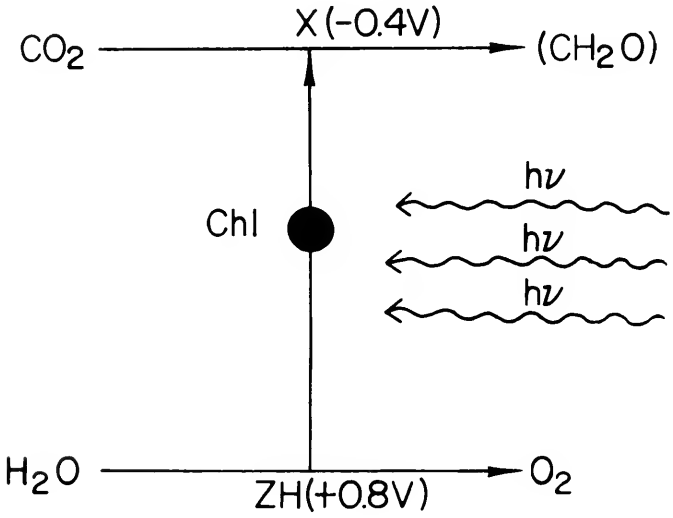

Fig. 1

The first two sets are "dark," enzymatic processes, occurring at an approximately constant level of energy, the third an energy-accumulating photochemical process. This latter must be an oxidation-reduction--either hydrogen atom transfer, or electron transfer, or a combination of both. This transfer goes from a donor, ZH (an intermediate in the

lower enzymatic reaction chain), with a high oxidation potential (approximately +0.8 V), to an acceptor (an intermediate in the upper enzymatic reaction chain) with a high reduction potential (approximately -0.4 V). It thus leads to the storage of about 1.2 eV of chemical energy per electron (or hydrogen atom) transferred. Since the reduction of carbon dioxide to the carbohydrate level requires the transfer of four hydrogen atoms, the total storage in the reduction of one carbon dioxide molecule is 4.8 eV, or about 110 kcal. per mole. Energy storage in a form other than oxidation-reduction energy can play only an auxiliary role in photosynthesis; this applies, in particular, to the formation of high energy phosphate (ATP).

2.

Experiments by Emerson (and others) have established that in normal photosynthesis, about 8 light quanta are needed to transfer 4 hydrogen atoms (or electrons) "from ZH to X" (in figure 1). As suggested already in 1947, this could be achieved in two ways: either by eight parallel one-electron transfers, followed by four dismutations; or by two consecutive sets of four transfers each. Various recent studies support the second alternative. They suggest that the first transfer leads from ZH to an intermediate acceptor roughly halfway between XH and Z--in other words, with an oxidation potential of the order of +0.2 V--and the second from this to the ultimate acceptor, X.

The finding of cytochromes in chloroplasts, due to Robin Hill and coworkers, led to speculations on the role of these catalytic proteins in photosynthesis. At first, they have been assigned--by Arnon, among others--to positions on one or the other end of the photochemical sequence. This required, however, ascribing to them an extreme (positive or negative) potential, not characteristic of known cytochromes. Much more plausible is Hill's and Bendel's recent suggestion that the chloroplast cytochromes serve as intermediates between the two photochemical reactions. Of the two cytochromes found by Hill in chloroplasts, one belongs to the group of cytochromes b, ("cytochrome b_6"), and has a normal potential of approximately 0.0 V, while the other, designated as "cytochrome f" (of cytochrome c type), has a potential of +0.37 V. It has been suggested that they serve in series, as indicated in figure 2.

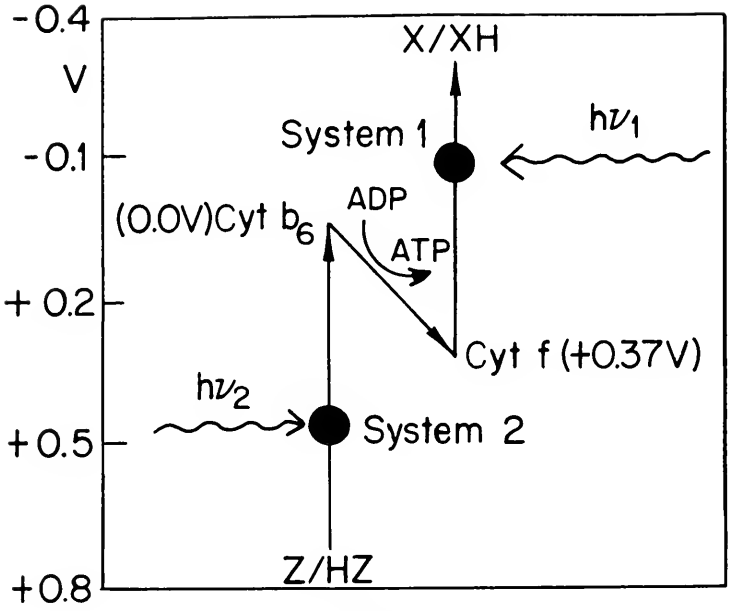

Fig. 2

Interestingly enough, two other oxidation-reduction systems have been identified recently in chloroplasts, one (plastoquinone) having a potential close to that of cytochrome $\underline{b}_6$, the other (plastocyanine) one close to that of cytochrome $\underline{f}$. Perhaps, they function as regulating systems, keeping the Fe^{3+}/Fe^{2+} ratio in the cytochrome systems stable. Another possible function of these quinones could be to provide transition from the "mono-valent" cytochromes to the "di-valent" redox systems at the beginning and end of the reaction sequence.

The first oxidation-reduction step, which transfers the hydrogen atom (or electron) from an oxidant, ZH, with a potential of about +0.8 V, to cytochrome $\underline{b}_6$ with a potential close to zero, stores about 0.8 eV (or 18.5 kcal. per mole) of the energy of the quantum. (The latter, in the case of chlorophyll $\underline{a}$, contains between 35 and 43 kcal. per mole). The photochemically reduced cytochrome b_6 then reduces, in a

nonphotochemical reaction, the cytochrome f, with a potential of +0.37 V, thus liberating about 8 kcal. of stored energy. This energy does not need to be lost; it seems plausible that, similarly to the electron transfer between cytochromes in the respiration chain, a part of it (about 7 kcal. per electron pair) can be salvaged in a molecule of high-energy phosphate, ATP.

The second photochemical reaction transfers the electron from cytochrome f to the acceptor, X, again storing about 0.75 eV of potential energy.

3.

Schemes similar to figure 2--presented vertically, horizontally, in zig-zags, on circles or curlicues--have been proposed by several authors in recent years. They have arrived at these schemes in different ways; the following appears to me as the logical sequence of findings leading to it:

a. Realization that in photosynthesis, four H-atoms (or electrons) have to be transferred "uphill", over a gap of 1.2 V.

b. Finding that 8 quanta are used in this transfer (Emerson).

c. Finding of two cytochromes in chloroplasts, with potentials suggesting their function as "halfway stations" in the transfer of electrons. (Hill).

d. Observations of the oxidation of cytochrome in chloroplasts by light absorbed in one "pigment system," and reduction by light absorbed in another "system" (Duysens).

e. Observation of an enhancement of the photosynthetic action of light absorbed in "system 1," by light absorbed in "system 2" (Emerson).

The last-named line of evidence appears to me particularly convincing. This matter will be presented in more detail in Govindjee's paper later in this symposium.

It is difficult to show that excitation of system 2 is by itself insufficient to bring about complete

photosynthesis, since photosynthesis is known to proceed, at high rate, in red algae, in 540 mμ-light, absorbed mostly by phycoerythrin. Two possible explanations may be given. In the first place, light absorption in system 2 could lead to resonance transfer of energy to system 1, thus achieving the required co-excitation of the two systems ("spilling over"). Secondly, the presence of some phycoerythrin in system 1 (suggested by Duysens) and the contribution of chlorophyll a to the absorption at 540 mμ, may be sufficient to achieve co-excitation of the two systems even without energy transfer. If the latter is true, the suggestion, presented further below, that the two systems may be contained in separate layers, would become more plausible.

Experiments by S. Brody, and by W. Butler and coworkers suggest that the fluorescence band of chlorophyll a *in vivo*, lies, at ordinary temperatures, at 680 mμ and belongs to chlorophyll a 670. It is thus associated with pigment system 2. (At the liquid air temperature, however, fluorescence bands appear also beyond 700 mμ--and must be attributed to chlorophyll a in system 1). The *primary* distinction between the two systems probably consists in their different location in the chloroplasts, implying association with different cellular components. Since chlorophyll molecules are known to fluoresce (in solution) only when associated with polar molecules, and to be non-fluorescent in dry hydrocarbons, one can suggest that pigment system 1 is associated with hydrophobic organic molecules, and system 2 with hydrophilic proteins.

It is known that chloroplasts consist of alternating lamellae of more hydrophilic and more hydrophobic nature. It is tempting to associate the fluorescent pigment system 2 with the hydrophilic, and the non-fluorescent pigment system 1 with the hydrophobic layers in the chloroplasts. One could, for example, postulate a *bimolecular sheet* of pigments, with one leaf turned towards the hydrophilic, aqueous layer, and the other towards the hydrophobic, lipoid layer; the two leafs could be separated by an enzymatic layer, containing *e.g.*, the two cytochromes.

Another alternative is to postulate a more uniform distribution of pigments in the two layers. The first alternative is supported, however, by Goedheer's optical findings, which suggest that chlorophyll in the chloroplasts forms separate lamellae less than 10 A thick. (The proteidic and

the lipoid layers having a thickness of the order of 30-50 A).

Another problem is the relationship between the layered structure, as observed on chloroplast sections, and the "cobblestone" appearance of separate lamellae when viewed from above. In the interpretation of the structure of mitochondria, the layered structure has been emphasized at some times, and the presence of, more or less spherical, enzymatic units, at other times. Perhaps, the lamellae in chloroplasts, similarly to those in mitochondria, also bear --or even themselves consist of--spherical units, perhaps identical with the "photosynthetic units," whose existence was first deduced from the experiments by Emerson and Arnold on photosynthesis in flashing light, and since supported by many other observations. Thomas observed that for chloroplast particles to be effective in the Hill reaction, they have to contain at least two layers--an observation that fits into the above-derived picture.

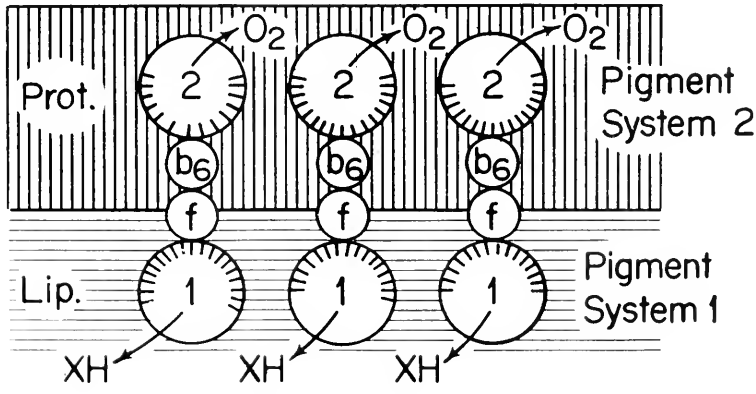

Fig. 3

Figure 3 represents a two-layer photochemical system. Within each layer, resonance transfer must take place both between identical pigment molecules, (leading to energy migration through the layer, at least within the confines of a single photosynthetic unit), and between pigments absorbing at the shorter wavelengths and those absorbing at the longer wavelengths. The energy quanta absorbed in each unit may be thus conveyed to a single enzymatic site. The chlorophyll molecules immediately associated with this site may be different in the two layers; their absorption band may be located at 700-710 mμ in the hydrophobic system 1, and nearer 670 mμ in the hydrophilic system 2. The question arises: How is it possible for the photoxidative process in one layer to be effectively coupled with a photoreductive process in the opposing layer, preventing the loss of energy by back reactions within the layer? Is the existence of photosynthetic units in both layers, with their enzymatic centers in juxtaposition, sufficient to insure effective correlation, evidenced by utilization of almost all light quanta absorbed, (at least, in weak light)?

In the light of Menke's results, one is tempted to equate the photosynthetic units in the hydrophilic layer with his "crystallites," and consider the possibility of chlorophyll molecules in the hydrophobic layer not being associated in units at all. However, bringing quanta absorbed by such scattered molecules into action at the proper reaction site, would be very difficult.

The effectiveness of coupling of events in two layers depends on the time interval allowed between the two photochemical reactions. The observations, by Myers and French, of the Emerson enhancement in flashing light suggest that the intermediates involved may be long-lived enough to permit effective coordination of the two processes, even in weak light. (Otherwise, the light curves, showing the rate of photosynthesis as function of light intensity, would be quadratic in weak light and only gradually approach linearity)!

4.

Another question is that of the mechanism of function of the sensitizing pigments. Do they themselves serve as reversible oxidation-reduction catalysts, (or more exactly, "photocatalysts") in the electron (or hydrogen) transfer chain? The search for evidence of reversible oxidation-reduction reactions of chlorophyll in the sensitization of

photosynthesis in vivo remains inconclusive. We know from the work of Krasnovsky, Evstigneev and coworkers, that, in vitro, chlorophyll sensitizes energy-storing oxidation-reductions, (such as that of riboflavin by ascorbic acid), by itself undergoing reversible reduction.

Measurement of difference spectra by Coleman (in our laboratory), and by Kok at RIAS, suggested that in sufficiently intense light, difference bands appear in the region of chlorophyll absorption, in the red as well as in the blue, indicative of reversible bleaching of chlorophyll, beginning when saturation of photosynthesis sets in. However, negative difference bands appearing in the neighborhood of 680-690 mμ, could be due to changes in fluorescence. (Fluorescence caused by the modulated measuring light beam could be made more intense by strong "background" of non-modulated actinic light; it is known that the yield of fluorescence in vivo is about doubled when cells pass from the "light-limited" into the "light-saturated" state). Coleman thought that he had disposed of these objections by re-routing the light beam under a right angle to its original direction, and decided that not more than 10% of the difference band he observed at 680 mμ could be due to fluorescence effects. More recently, however, Mr. Rubinstein in our laboratory found--by measurements in polarized light--that a much larger proportion, (perhaps, the whole) of the negative difference bands observed at 680 mμ, were in fact due to fluorescence changes. Independently, similar conclusions were reached by Krasnovsky and coworkers.

However, certain true changes in absorption spectra in the chlorophyll a absorption region, do occur; they include a "negative" band at 648 mμ, and a "positive" one at 658 mμ. These bands were first observed by Strehler, and also noted by Coleman. Recently, they were studied much more precisely by Rubinstein in our laboratory. Their occurence suggests that some reversible photochemical transformations do occur in the chlorophyll system during photosynthesis; but whether these are in the nature of oxidation-reductions (such as would be expected if chlorophylls were to serve as a wayside station in the transfer of electrons or H-atoms in photosynthesis) remains uncertain. Kok found a difference band at about 705 mμ, which he attributed to reversible photochemical transformation of a long-wave component of the chlorophyll system directly associated with an enzymatic center and serving as a final "sink" in the energy trans-

fer chain. In this case, too, the possibility of this change being in the nature of oxidation-reduction remains uncertain. Witt considered certain difference bands he observed in the short-wave region of the spectrum as indicative of reversible oxidation and reduction of chlorophyll, but this interpretation, too, remains hypothetical.

The old problem of chemical (photocatalytical) versus purely "physical" sensitizing action of chlorophyll in photosynthesis, thus remains an elusive one.

5.

Two types of fluorescence measurements need to be interpreted for the two-layer pigment picture to be convincing. One is the above-mentioned finding, by Franck and coworkers, (as well as by Wassink and others), that the fluorescence yield of photosynthesis doubles (from about 1.5% to about 3%) when photosynthesis becomes light-saturated.

In our picture, chlorophyll is supposed to fluoresce in the hydrophilic layer, where it sensitizes the oxidation of water, and to be non-fluorescent in the hydrophobic layer, where it sensitizes the reduction of the organic substrate, X. Additional *ad hoc* hypotheses are needed to explain in this picture why the fluorescence yield should double at light saturation of photosynthesis. (Franck sees in this finding the evidence that of the two photochemical steps, one is brought about by fluorescent, and one by metastable chlorophyll molecules).

The other finding is due to S. Brody, who found in our laboratory that the *lifetime* of chlorophyll fluorescence in living cells is slightly less than one half of that in solution--while the steady fluorescence *yield* is at least 10 times smaller (3% vs. 30%). Brody's value was subsequently confirmed by more precise measurements of Tomita and Murty. They support the presence *in vivo* of two forms of chlorophyll, but suggest that the fluorescent form accounts for only 20% of the total, and the non-fluorescent for 80%. However, Brody's results were obtained by means of very brief flashes of illumination; the *average* light intensity was very low, and the cells could be presumed to remain in the dark-adapted state. Lifetime measurements should be repeated with cells illuminated with a background light, sufficiently strong to maintain steady photosynthesis.

Obviously, more studies are needed before it could be said that the fluorescence data confirm (or contradict) the two-layer scheme, suggested in this paper.

6.

The lamellar structure of the photosynthetic apparatus in general--and the two-leaf structure discussed here in particular--may favor effective storage of light energy in two ways: (1) by permitting resonance energy migration within the layers, and thus facilitating the utilization of quanta absorbed in a large number of chlorophyll molecules, for chemical transformations in a--by geometrical necessity, much smaller--number of enzymatic sites; and (2) by causing spatial separation of the oxidation and reduction products in two different layers, thus making immediate back reactions unlikely. (For example, the organic reduction products may be given off into a hydrophobic, lipoid layer, while the photoperoxides, and ultimately, oxygen, are evolved into the hydrophilic, proteidic layer). In this connection, I would like to mention one experiment. On my suggestion, Dr. Mathai carried out the oxidation-reduction reaction between ferrous iron and thionine dye in a two-phase system, (ether + water). This reaction is in some respect analogous to photosynthesis, or even more, to Krasnovsky's reaction between chlorophyll and ascorbic acid; it stores energy equivalent to a potential difference of about 0.3 V, by using visible light energy, absorbed in a dye molecule. When illumination stops, the reaction is reversed and the color of the dye returns. When this reaction is carried out in a suspension of ether droplets in water, the leucothionine is extracted into the ether, while the ferric ions stay in the aqueous phase; the two phases could be separated in a funnel, and both remain colorless. But when alcohol is added, permitting the two phases to mix, the delayed back reaction takes place, and the mixture immediately becomes dark purple. This simple experiment demonstrates the effectiveness of phase separation of products for chemical storage of light energy in an oxidation-reduction system.

FLUORESCENCE IN TWO-PIGMENT SYSTEMS

J. L. Rosenberg and Tevfik Bigat

We report here on some of our recent experimental observations of fluorescence in algae and leaves, with particular reference to the interactions between the two pigment systems and between the two photochemical reactions of photosynthesis. A unified model for energy collection and for the photochemical sequence of photosynthesis will be assumed, applicable to green algae, phycobilin-containing algae, and green leaves.

RED ALGAE

Experimental

Fluorescence observations of Porphyridium cruentum were carried out under conditions of chromatic transients occurring when the wave-length of illumination is changed. In addition steady-state intensities of fluorescence were observed under differential excitation of the chlorophyll a and of the phycobilins. Duysens' publication of a similar study appeared while our own manuscript was in preparation[1]. We therefore need not give great experimental detail, but mention particularly those points of difference between our work and his. We will use his notation wherever possible.

The density of the cell suspension was such as to allow 25-30% transmission at 680 mμ in the 1 mm optical path of the fluorescence cell. The sample could be illuminated with two beams entering the cell along the same path, a blue beam at 447 mμ absorbed principally by chlorophyll a and a green beam peaking at 535 mμ absorbed principally by phycoerythrin. These beams served both as actinic and fluorescence excitation beams. Fluorescence was observed at 45° to the actinic beam through a 679 mμ interference filter appropriately supplemented to eliminate all scattering and phycobilin fluorescence.

For approximately equal incident intensities of the two beams, the steady-state fluorescence during simultaneous irradiation was

not only less than the sum of the fluorescences with separate excitations but was less than the fluorescence with the green beam only. In a typical experiment, for which the green beam intensity was 97 and the blue 90, in units of 10^{-11} einstein/cm^2 sec, the relative steady-state fluorescence intensities for green, blue, and green plus blue excitations were 130, 25, and 100 respectively. The attainment of these steady values in changing from one type of illumination to another was achieved by way of interesting transients. Fig. 1 summarizes the transients accompanying addition or removal of blue excitation to a sample under continued green irradiation. The transient

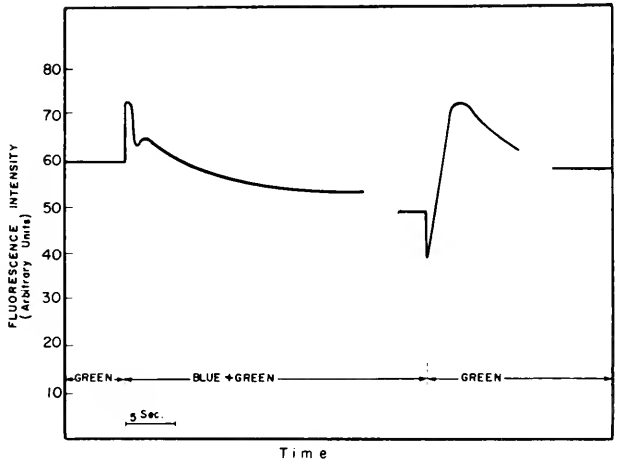

Fig. 1. Chlorophyll a fluorescence of Porphyridium showing transients due to shifting from green to green plus blue illumination and the reverse. The regime of illumination was not changed until the steady-state of fluorescence was reached for each type of illumination. Incident intensity for the green beam was 97 and for the blue, 90 (in 10^{-11} einstein/cm^2-sec).

accompanying the shift from blue to green excitation is shown in Fig. 2. Here the initial instantaneous rise in fluorescence is followed by a slower sigmoid rise to a new steady-state at a rate increasing with increasing green light intensity. We failed to observe the maximum found by Duysens for Porphyridium but not found by him to a large extent in other species. We

believe that his maximum might be a normal induction outburst, and that its occurrence might require a longer elapsed period at low photosynthetic activity (dark or blue light) before switching to green than was allowed in our experiments.

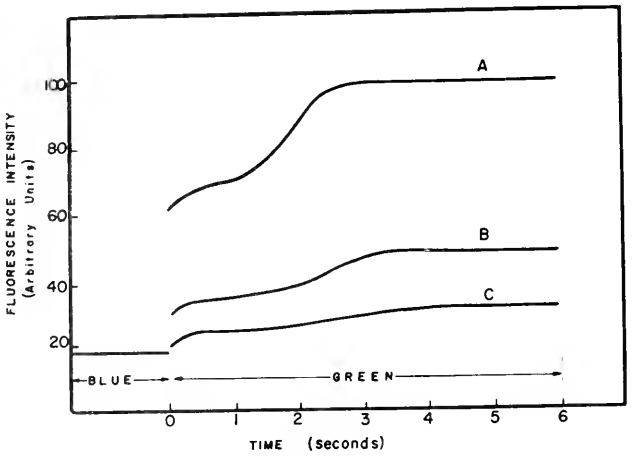

Fig. 2. Chlorophyll a fluorescence of Porphyridium showing the chromatic transient accompanying the shift from blue excitation (I = 128) to green. Intensity of green, in units of 10^{-11} einstein/cm^2-sec: A, I = 158; B, I = 79; C, I = 40.

Discussion

The above results, like those of Duysens with red algae[1] and of Govindjee et al. with Chlorella[2], may be interpreted on the basis of two pigment systems. System 1, containing the bulk of the chlorophyll a in the red algae and the long-wave absorbing component in the green algae, gives very little chlorophyll a fluorescence; while System 2, containing the phycobilins in the red algae and the bulk of the chlorophyll a in the green algae, is capable of a strong chlorophyll a fluorescence.

To accommodate the experimental results we start with a model different from that of Duysens in one very important respect. He postulates that System 1 is required for the sensitization of photochemical Step (1) and that System 2 can sensitize only photochemical Step (2). The substrate for Step (2), Q is

an oxidized direct or indirect product of Step (1) and is reduced photochemically by the fluorescent state of System 2. We base our interpretation on the model of Franck and Rosenberg,[3] in which the non-fluorescent System 1 efficiently sensitizes only Step (1), as in Duysens' scheme, but in which System 2 may sensitize either Step (1) or Step (2), Step (1) by way of a metastable state and accompanying fluorescence and Step (2) by way of the excited singlet state without fluorescence. With this difference we propose the following correlations with experiment:

(a) The action spectrum determined by Duysens for photosynthesis against a background of strong green light,[1] similar to the action spectrum for cytochrome oxidation in the presence of DCMU,[4] shows equal effectiveness of chlorophyll a and phycoerythrin. We assign this as the action spectrum for Step (1), not System 1, and expect it to be a combined action spectrum for System 1 plus System 2.

(b) System 2 is capable of efficient photosynthesis by itself. This correlates with the observed proportionality between the action spectra for photosynthesis and for chlorophyll a fluorescence.[5] Although good absolute quantum yield data may not exist showing a minimum quantum requirement of 8 for red algal photosynthesis, there certainly is good evidence with Chlorella and with brown and blue algae that the minimum quantum requirement may be achieved with monochromatic light over a wide wavelength region.[6] If the same distribution of function between two pigment systems exists in all the algae, this fact could be explained only if one of the systems may sensitize both photochemical steps.

(c) A long-wave absorbing pigment, like P700, cannot act as a sink for singlet excitation energy during normal photosynthesis under illumination absorbed by System 2, even when System 2 is being used for Step (1). If we assume that P700 removes excitation in connection with Step (1), then the observed chlorophyll a fluorescence would have to arise by competition with Step (2). This is in contradiction to the finding in our experiments and in those of others that the fluorescence from System 2 is depressed particularly when this system is working with high efficiency in sensitizing Step (2), namely, when simultaneous blue irradiation provides adequate Q substrate. An attempt to retain the assumption of trapping by P700 by allowing a small fluorescence leak during the flow of excitation from System 2 to P700 would not work, because a small fluorescence leak of chlorophyll a is always accompanied by a many-fold greater leak into the

metastable state of chlorophyll a, and the combined energy losses for fluorescence and metastable state formation would be too excessive to allow for good quantum yields.

(d) Existing fluorescence data need not be interpreted to exclude the possibility that Q, the substrate for Step (2) is oxidized cytochrome f. This exclusion has been made by Duysens[1] and in somewhat different language by Kautsky et al.[7] on the ground that the fluorescence is low after a dark period when cytochrome f is known to be reduced. It must be emphasized that oxidized Q is not the only quencher of chlorophyll a fluorescence. It is well known that after a dark period metabolic oxidants substitute for the normal primary photosynthetic oxidants during the beginning of the induction period.[8] Even on steady irradiation at intensities below compensation this type of substitution occurs and is accompanied by lower than normal fluorescence yields.[9] The lowering of fluorescence under such conditions can be explained in terms of a postulated adsorption of these substitute oxidants at the chlorophyll and their consequent ability to use chlorophyll a singlet excitation directly in a variant of Step (1), thus quenching fluorescence.[10]

(e) Since the above model allows System 2 to sensitize both photochemical steps, it is necessary for reasons of geometrical economy about the bifunctional reaction center that there not be too many different photochemical substrates that are part of the built-in photosynthetic apparatus. For this reason, as well as for others discussed elsewhere in this Symposium,[3] we propose that Q, the normal quenching substrate for Step (2), is oxidized cytochrome f and that the same cytochrome in the reduced form is a substrate for Step (1).

(f) In the absence of substitute reactions discussed in (d) the chlorophyll a fluorescence yield in the light-limiting region depends mainly on the ratio, r, of the rate of use of System 2 for Step (1) to its use for Step (2). The shape of the transients shown in Figs. 1 and 2 can be accounted for very well in terms of the adjustment of the value of r to the illumination regime, with the detailed model of Franck and Rosenberg predicting that r may range from a value close to zero, immediately after a period of System 1 light absorption and oxidized cytochrome accumulation, to a value of unity during steady-state absorption by System 2 alone.[3]

J. L. Rosenberg and Tevfik Bigat

GREEN PLANTS

We report here two experiments with green plants which bear on the problem of interaction between the two photochemical systems. By a procedure described in detail elsewhere, we determined the fluorescence spectrum at the one-second peak of the induction outburst following a dark period relative to the spectrum at steady-state irradiation. Fig. 3 summarizes some typical results.

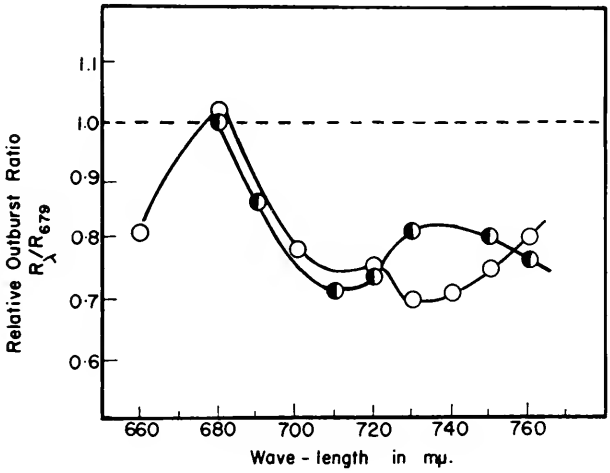

Fig. 3. Relative fluorescence spectrum of a fresh Forsythia leaf at the induction maximum. The leaf was illuminated in air with blue-green light, I = 800 x 10^{-11} einstein/cm^2-sec, following a 10-minute dark period. R_λ is intensity of emission at wave-length λ at the steady-state, reached after about 20 seconds of irradiation. The abscissa, measuring the ratio of R_λ to R_{679} (using 679 mμ as a reference wave-length), would follow the dashed horizontal line if the induction outburst had the same spectrum as the steady-state fluorescence. The two curves are for different samples.(10)

The curves of Fig. 3 show that there is relatively more increase in 679 mμ fluorescence (the main chlorophyll a band) during the outburst than in the longer wave-length component. The same was found for many kinds of samples, both leaves and

algae. These results are similar to those reported by Lavorel for Chlorella by a completely different method,[11] and are consistent with the finding of Butler that there was practically no increase in the ability of light at wave-lengths above 700 mμ to excite fluorescence at the induction outburst.[12]

The simplest interpretation of these facts is that the far-red fluorescing pigment, corresponding to P700 in absorption, can participate in photochemistry only indirectly or only by way of its metastable state. If this pigment belongs to System 1 and acts as an energy sink for other pigments within System 1, there would be a unique assignment of the photosensitizing role for Step (1) to a metastable state, whether System (1) or System (2) absorption acts lead to this photochemical act.

Fig. 4 shows the results of some typical fluorescence transients in leaves accompanying the reduction of incident intensity from a value above saturation to a lower value. These experiments were a confirmation and extension of a phenomenon reported by Franck et al.[13]. The different courses of Curves A and B in Fig. 4 suggests a difference in mechanism of saturation for the aerated and CO_2-depleted samples. In the aerated leaf, curve A of Fig. 4, the major cause of saturation is probably located in the oxygen-liberating reactions following Step (2). Under such conditions oxidized cytochrome accumulates but is not able to undergo Step (2) as rapidly as quanta are supplied to the reaction centers. When the incident intensity is lowered, a region of greater quantal utilization is reached. Efficient photochemistry will start with Step (2) to reduce the backlog of oxidized cytochrome. The fluorescence yield will thus start low at the reduced intensity and will slowly rise as the excess oxidized cytochrome is reduced and the two photochemical steps again come into phase with each other.

In the CO_2-deprived sample, Curve B of Fig. 4, the major cause of saturation is the limitation in supply of the natural photosynthetic oxidant for Step (1). In such a case, prolonged irradiation at high light leaves the cytochrome principally in the reduced state. On a sudden reduction in light intensity a more efficient quantal utilization will be felt primarily in Step (1), and the fluorescence will remain above the final steady-state value until the two photochemical

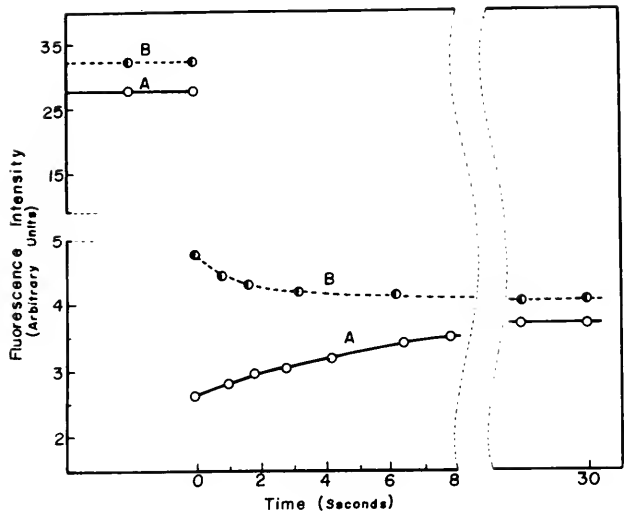

Fig. 4. Fluorescence at 680 mμ of a bean leaf following the transition from high light to low light irradiation. Incident intensity in blue-green prior to zero time, in units of 10^{-11} einstein/cm^2-sec: A, I = 336, B, I = 250. Intensity was reduced to one-eighth initial intensity at zero time. A: leaf in moist air; B, leaf in CO_2-free air.(10)

steps come into phase with each other. The above explanation may not include all factors responsible for the transients in Fig. 4 but does provide a basis for understanding the difference between the two cases studied.

ACKNOWLEDGMENTS

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RELATIONSHIP BETWEEN LIGHT INDUCED EPR SIGNAL AND PIGMENT P700

Helmut Beinert and Bessel Kok

In two previous communications (1,2) we have reported observations on the narrow (~8 gauss) EPR signal induced by light in photosynthetic materials. (This signal is also known as signal I (Ref. 3) or signal II (Ref. 4) and rapidly decaying or "R" signal (Ref. 5). The first paper (1) described properties of the signal observed in a preparation of the red alga strain TX 27 that was largely deprived of phycobilin and enriched in "P700" by partial extraction of the chlorophyll using 72% acetone (6). The signal appeared on illumination at room or liquid nitrogen temperature or after chemical oxidation by means of ferricyanide. Double integration of the derivative signal indicated a concentration of spins nearly identical to the concentration of P700 as determined by difference spectroscopy assuming a molar extinction of 10^{-5} $M^{-1}xcm^{-1}$ and complete bleaching in the photoact. A second extraction of the algal preparation, now with 80% instead of 72% acetone, removed the EPR signal as well as the difference spectroscopic signal at 700 mu. We concluded that these results could be most readily explained if the photooxidized form of P700 was responsible for the narrow, fast decaying EPR signal in photosynthesis.

The second paper (2) reported observations with whole cells of the blue-green alga Anacystis at room temperature. The relative strength of the narrow EPR signal was measured as a function of intensity of either one or both of two wavelengths: 630 mu absorbed by phycocyanin and quite effective in provoking photosynthesis and 710 mu absorbed by (long wave) chlorophyll and rather ineffective in provoking photosynthesis. The EPR signal showed the same behavior as the oxidized form of P700 which was observed earlier spectroscopically (7): Long wave light proved much more effective in provoking the EPR signal than short wavelengths, or a combination of the two lights. These data therefore also indicated the possible identity of the EPR signal with oxidized P700, presumably the oxidized moiety generated in the long wave photoreaction of photosynthesis. Since its reduction requires the reduced moiety generated in the short wave photoact, $P700^{+}$ tends to accumulate in the absence of the short wave light.

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Weaver and Bishop (8) recently failed to observe the narrow EPR signal in a Scenedesmus mutant (#8). Spectroscopic examination of this alga indeed did not reveal a light induced bleaching of P700 (9).

Spectroscopic determination of P700 revealed a maximum absorbancy change at 700 mu of 1 unit per 300-400 units of total chlorophyll absorbancy (at ~675 mu). Its soret band (at 432 mu) as well as its nearly identical solubility in organic solvents indicate P700 is a chlorophyll a molecule. We assume that a special binding site causes a long wave change of its absorption band and underlies its function as a photoconverter. This assumption assigns to P700 a molar extinction coefficient similar to that of chlorophyll a ($\sim 10^5$ *in vivo*).

A second assumption: that complete bleaching occurs in the photoact yields a ratio of one trapping center per 300-400 sensitizing chlorophyll molecules--in good agreement with measurements of the "photosynthetic unit" (10,11).

If the oxidized form of P700, $P700^+$, which is formed in the long wavelength photoact, were identical with the free radical species observed by EPR, the quantitative relationship, between the number of spins represented by the EPR signal and the amount of P700 detected optically, which we observed in a preliminary experiment (1), should hold rather generally for photosynthetic materials and furthermore, the kinetics of appearance and disappearance of the EPR signal should match that of the typical absorption band of P700.

In the present work we undertook a direct approach at quantitation of the number of spins represented by the light induced EPR signal and the amount of chlorophyll and P700 present in a variety of materials. Knowledge of any consistent quantitative relationships would obviously be of interest, even if the EPR signal were not due to $P700^+$ itself or a closely associated electron carrier or trap.

We were aware during the course of this work that quantitation of EPR signals is beset with many difficulties, that we had to make certain assumptions, which are not readily amenable to experimental verification, and that a definitive identification of the component responsible for the EPR signal could not be expected from our approach. The EPR data were evaluated on the assumption that the light induced free

radical species are fully detectable by EPR and that no interactions interfere with this detectability.

The concentration of unpaired electrons was measured by EPR spectroscopy at ambient and low (-50° to -70°) temperature as will be described in detail elsewhere (12). For the experiments at room temperature a double cavity was used, which held both sample and standard in matched flat cells. A benzene solution of diphenylpicrylhydrazyl (DPPH) was used as a standard at room temperature and a pitch sample in KCl and nitrosyldisulfonate in KOH at low temperature. The standards as well as the integration procedure used were cross checked. Conditions were chosen that saturation of the EPR signals with microwave power did not occur or was sufficiently small that it could be corrected. In order to ensure saturation of the pigment suspension with light, i.e. maximal signal development, the signal amplitude of serial dilutions of these suspensions was measured until a linear relationship between signal amplitude and concentration was observed. The double integration of the derivative signals, which resulted in the quantitative estimate of unpaired spins, was based on signals obtained in this linear region. Values were also observed in the dark and after addition of ferricyanide. The value obtained with ferricyanide can in many cases serve as a control for light saturation and maximal signal development, as the concentration of unpaired electrons produced by an excess of this oxidant is either very similar to that obtained on illumination under saturating conditions or larger. Only materials were selected for study in which overlapping signals (broad signal, 20 gauss, slow decaying or "S" signal, and Mn (II) signal) were absent or small. Overlap, when occurring, was corrected.

Results obtained under satisfactory experimental conditions are summarized in the table. The last vertical column gives the calculated ratio of unpaired spins per P700. Most values cluster around a ratio of 2, although the low temperature experiments on the TX 27 preparations and the experiments on Anacystis yielded higher ratios. We have no explanation for the high values obtained at low temperature. Although a different standard (pitch) was used at low temperature than at room temperature, a comparison of both standards gave excellent agreement. It is likely that the high values obtained for whole Anacystis and the same preparation after sound treatment are due to overlap of the narrow EPR signal with the broad light induced EPR signal in such

complex preparations. Although overlap was corrected on the basis of the dark signals observed after illumination, this correction is not entirely satisfactory as the broad signal is more intense during illumination. In the last two lines determinations on chromatophores from Rhodospirillum rubrum are reported. Since P700 is not a constituent of these organisms only the ratio of bacterio chlorophyll [determined according to (13)] to unpaired spins is given. Approximately 3% of this chlorophyll are thought to represent a photoconverter, P890, similar to P700. On this basis a spin per P890 ratio of 0.4 to 0.5 would be obtained.

We are aware that our experiments cannot provide a final decision or an identification of the narrow light induced EPR signal; they could at best rule out or make appear plausible certain possible interpretations. In assessing the significance of the values we obtained, two principal considerations are pertinent: The first is concerned with the accuracy of our quantitation procedures and the second with the question of whether all radicals and radical species formed in the illuminated samples were, in fact, detected by EPR.

To the first point we can say that use of the double sample cavity and carefully matched cells, the use of independently standardized standards, attention to the conditions of saturation with light and microwave power and the consistency of the results obtained in the determinations at both room and low temperature, make it very unlikely that gross errors were committed. Nevertheless, in view of the uncertainties in the absolute values of EPR standards and in comparison of different materials, we think that accumulation of errors could have led to values which are in error by a factor of 2 or 3. The consistancy of the results indicates that these errors, if incurred, are not random but systematic and due to certain incorrect assumptions.

The second point of concern is related to the question as to what type of paramagnetic species is in fact responsible for the observed signal. The simplest assumption, on which our experiments here are based, is that a single free radical species arises, which has a structure and environment such that it can be quantitatively detected by the EPR technique. However, since a one electron oxidation produces the radical, it appears possible that a second radical, formed by the corresponding one electron reduction (in the extreme case a free electron) is simultaneously generated. There are no

indications we know of from the behavior of the observed EPR signal that it may represent two different species. This would still not exclude such a possibility, for which one could see some support in the high ratios of free spins produced per molecule of P700 (cf. Table 1).

A serious objection to the interpretation of our experimental results could arise from the possibility that we might not be detecting more than a fraction of the radicals actually produced. Several explanations could be given for this. We may be dealing with "lifetime" broadening, i.e., short relaxation time of the unpaired electron; an exchange interaction may broaden the line; or, in case a free electron were generated, it could be trapped at non-equivalent sites and therefore, experience varying local magnetic influences, which would lead to line broadening. Such arguments cannot at present, be refuted on experimental grounds. If they were valid our values would set the lower limit of radical concentration.

We may then conclude from our data in the light of these considerations that the number of unpaired electrons induced by light in the photosynthetic material studied is either closely similar or bigger, certainly not smaller than the amount of P700 present. It is of interest to note that other components of the photosynthetic system in plants, have been reported to occur at a concentration of the same range as P700 and the light induced EPR signal studied here. However, the metal constituents of two of these: cytochrome f and plastocyanin are certainly not responsible for this signal.

ACKNOWLEDGEMENTS

We are indebted to Dr. J. Heise and Mr. R. W. Treharne of the Kettering Laboratories, Yellow Springs, Ohio, for their kind collaboration in the experiments with the double sample cavity and to Mrs. I. Harris for technical assistance.

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Table 1

Concentrations of Chlorophyll, P700 and Detectable Light-Induced Free Radicals in Various Photosynthetic Materials

Material	Temp. °C	Chlorophyll 10^{-5}M	P_{700} 10^{-7}M	Spins 10^{-7}M	Ratio: Chlorophyll per spins	Ratio: Spins per P_{700}
TX 27	25	72	38	67	108	1.8
broken, washed	-53	2.5	1.3	4.3	58	3.3
TX 27, washed	25	50	65	110	45	1.7
acetone extracted	-53	2.0	2.6	9.0	22	3.5
Anacystis	25	120	40	107	112	2.7
whole cells	-53	6	2	11	55	5.5
Anacystis	25	52	17	65	80	3.8
broken, washed	-53	2.7	0.9	3.8	71	4.2
Chloroplasts						
acetone extracted	-72	24	35	79	30	2.3
Chloroplasts						
fresh	-70	100	25	51	196	2.0
aged	-70	72	18	38	190	2.1
acetone extracted	-70	16	23	58	28	2.6
R.rubrum	25	210		330	64	
	-53	5.3		6.3	84	

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EPR AND OPTICAL STUDIES ON SCENEDESMUS MUTANTS

Ellen C. Weaver and Norman I. Bishop

There is more than one way to obtain information on single electron transfers as they are taking place in an illuminated photosynthetic system. One which we have been using for some time is electron paramagnetic resonance (EPR) spectroscopy[1]. There are two light induced resonances which are typical for the chloroplasts of higher plants and several species of algae. They can be differentiated on the basis of g-value, line shape, and the kinetics of their formation and decay. The most prominent one forms and decays in less than a second; hence the designation R, for "rapidly decaying". It has a g-value of 2.0025, is about eight gauss wide, is unstructured and Gaussian in shape. It has been shown to be dependent on the presence of chlorophyll[1,2]. The other resonance forms rather quickly, but persists in the absence of illumination for periods up to an hour or more; hence the designation S, for "slowly decaying". It has a somewhat higher g-value, 2.0046, is twenty gauss wide, and displays partially resolved hyperfine structure. It has been tentatively identified with the semiquinone of plastoquinone[3].

There are two classes of mutants which have provided further evidence on the role of the two EPR signals in photosynthesis[4]. Thesepossess all the readily identifiable wild type pigments in normal quantities, and yet are unable to photosynthesize. Those in one class are termed "oxygen" mutants, because, although they are able to photoreduce carbon dioxide with hydrogen, have a greatly reduced quinone-Hill reaction. All of these have a typical R signal, but display only a trace of the persistant S signal. This observation, together with that on manganese-deficient cultures, which also lack both Hill reactivity and the S signal[3], and photosynthetic bacteria which evolve no oxygen and also lack the S signal, enables one to identify the broad, structured signal with the ability of the system to evolve oxygen in photosynthesis, although other interpretations have been proposed[2]. They all possess the normal distribution of plastoquinones; some other essential link in the electron transport chain, not directly observable with EPR spectroscopy, is missing.

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The second class are termed "CO_2" mutants because they have a greatly reduced ability to photoreduce carbon dioxide with hydrogen, although the quinone-Hill reaction is relatively intact. One of these, Mutant 8, shows no R signal at all, even though it has abundant chlorophyll (4). Dr. Warren Butler's investigations on this mutant (5) indicate that it lacks P700, but information on this point for the other "CO_2" mutants is lacking. Fig. 1 illustrates the behavior of the cultures under consideration.

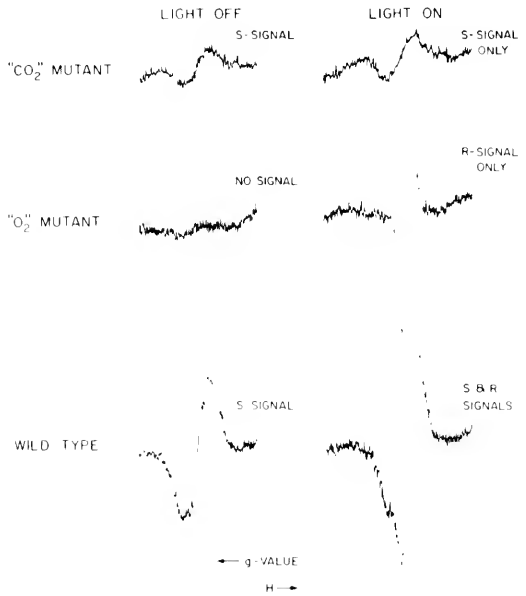

Fig. 1 Comparison of the EPR spectra in mutant and wild type Scenedesmus. Due to its long decay time, the S signal can be seen in the absence of illumination, whereas the R signal decays within seconds. g-value may be thought of as the EPR analogue of wavelength as used in optical spectroscopy. All spectra are made with suspensions containing approximately 5 x 10^8 cells per milliliter and with identical instrumental parameters.

Another "CO_2" mutant, No. 18, has a trace of R signal when exposed to bright light, but this may reflect the somewhat higher overall rate of photosynthesis of which it is capable. However, several other "CO_2" mutants have an R signal of approximately wild type proportions. Although a system capable of carrying out photoreduction can produce an R signal, the converse is evidently not true; and R signal is not a reliable indicator of photoreduction.

We were fortunate, therefore, to be able to compare the behavior of the mutants by optical means with the results already on hand. The difference spectrophotometer in Prof. Melvin Calvin's laboratory is somewhat similar to the one described by Dr. Bessel Kok[6] in that it makes use of repetitive flashes of actinic light. A spinning disc with a sector removed provides alternating light and dark periods. The absorption is measured at a given wavelength by a photomultiplier immediately after the flash, and again just before the flash; the difference in the absorption (light minus dark) is plotted against wavelength as the monochromator is slowly driven. This particular machine is described in detail in a forthcoming paper[7]. The spectra displayed here utilize a flash of 3 msec followed by a dark period of 30 msec; thus, only reversible changes with time constants which fall within these limits are detectable.

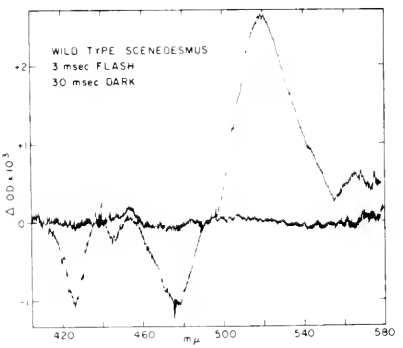

Fig. 2 Changes in absorption induced by 3 msec flashes in a suspension of Scenedesmus. The absorption was measured 4 msec after the onset of the flash, and again at the end of the 30 msec dark period. A tungsten lamp was used with a Corning 2030 filter passing only the wavelengths between 648 mμ and 750 mμ. The curve seen here is the first minus the second measurement, (light minus dark). The base line is the absorption just before the flash.

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The difference spectrum of wild type Scenedesmus, agrees in its major features with other published difference spectra for this organism[8]. In contrast is the spectrum of Mutant 8, a "CO_2" mutant, which displays virtually no spectral shifts; yet Mutant 18, in all other respects quite similar to Mutant 8, has a difference spectrum which resembles wild type (Fig. 3). Among the "O_2" mutants the same situation holds true: some (e.g. No. 11) have no spectral shifts, while others (e.g. No. 40) look very much like wild type. There is no obvious correlation between either of the EPR signals and any of the prominent positive or negative shifts in the region we were able to scan.

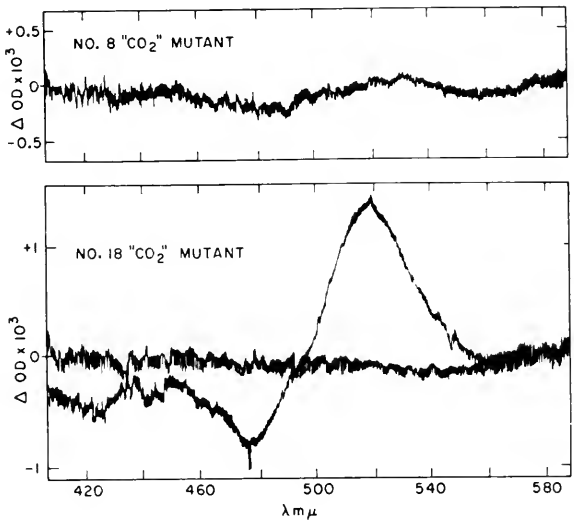

Fig. 3 Changes in absorption determined as in Fig. 2. The upper trace is that of No. 8, a "CO_2" mutant, and displays almost no changes. No. 11, an "O_2" mutant, is similar in its lack of signal. The lower trace is of another "O_2" mutant, No. 18; the "O_2" mutant No. 40 resembles it.

Fortunately, it was also possible to examine the time course of an absorption change at any one wavelength by using the machine in the following way: a neon flash bulb was substituted for the chopper, allowing the time sequence of light and dark to be completely controlled. With the very short time constants employed, sensitivity had to be sacrificed. This difficulty was circumvented by use of a computer of average transients (CAT); this device 're-

members' the signal from each of a large number of repetitions of each light and dark sequence. Noise, being random, is averaged out, while any consistent signal, however small, is built up to recognizable proportions.

We devoted most of our attention to the shift at 520 mμ; the 478 mμ shift seems to be related in a direct way to it in that it behaves in a similar way with varying conditions. Fig. 4a is a trace of the rise and decay of this shift in wild type cells; both curves are approximately exponential with the 0.1 second flash employed here. The whole cycle, light plus dark, is two seconds in duration. Fig. 4b at once tells us why we were seeing no shifts with the 3 msec flash in Mutant 11; although the positive shift is rapid, the decay is very much slowed down. If the absorption change is not reversible, the repetitive flash device will detect no change. For Number 8, (Fig. 4c) both rise and decay of the absorption shift at 525 mμ are slow. However, both

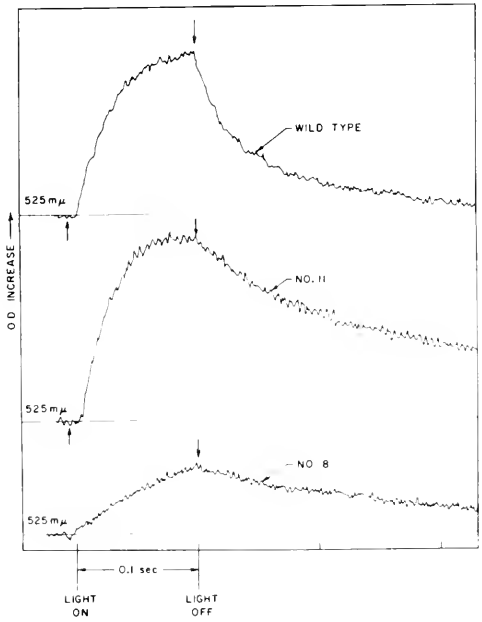

Fig. 4 Time course of the absorption change at 525 mμ produced by a neon flash in wild type and mutant Scenedesmus. Each curve is the summation of 100 repetitions of the light-dark cycle, made with the aid of a CAT (see text).

this "CO_2" mutant and the one most like it, Mutant 18, are capable of large shifts in absorption at 525 mμ if the light period is long enough. Figure 5 demonstrates that the absorption in No. 8 is still increasing, even at the end of a flash 0.4 second long.

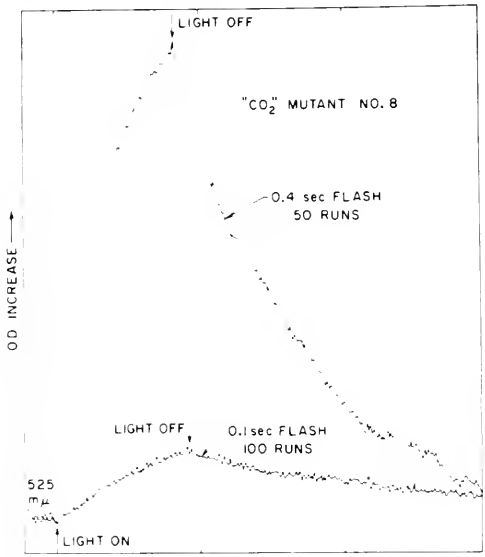

Fig. 5 Comparison of the effect of a 0.1 second flash in a total period of 2 seconds, which produces very little change in the 525 mμ absorption, with a 0.4 second flash (period 4 seconds) which produces a large change.

The picture is quite different when wild type is subjected to flashes of 0.5 second in length. There is a two phase rise at 525 mμ; first a very steep one, lasting just over 0.1 second, followed by a slower increase in absorption. Evidently at least two absorption changes, one considerably slower than the other, are taking place. The time course of the 425 mμ shift, however, remains exponential. If one observes the behavior of a representative "O_2" mutant, No. 11, with a 0.5 second flash, the time course is more complicated (Fig. 6). There is a steep rise, followed by a decay which sets in while the light is still on; further decay takes place when the light is turned off. It is almost as though some substance present in this mutant was decreasing in optical density at 525 mμ, but with a slower time course than the positive shift. Possibly this substance does not decay

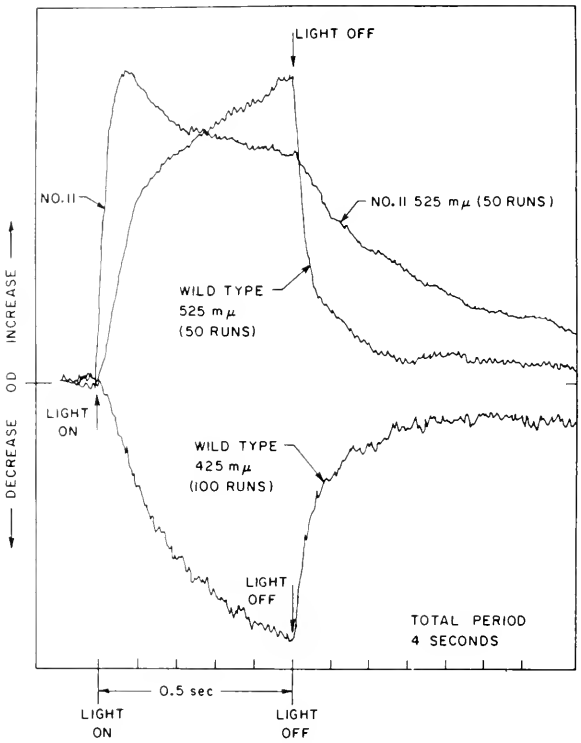

Fig. 6 Time course of absorption changes with a 0.5 second flash at 525 mμ in wild type and an "O_2" mutant (No. 11). The two phase rise in wild type is contrasted with the negative shift in the same preparation at 425 mμ which remains approximately exponential. Note the decay during the 0.5 second flash in No. 11.

entirely in the dark period, thus accounting for the greater initial increase in absorption observed with the shorter flash (Fig. 7).

The time course behavior of No. 8, wild type, and No. 11 compose a series of increasing complexity. These were suspended in water without added substrate. However, addition of benzoquinone to No. 8, while allowing it to evolve oxygen, does not perceptibly change its optical behavior. Nor does saturating No. 11 with hydrogen gas, which it can use for the photoreduction of carbonate, change the characteristics displayed here. However, it has been shown that adapting wild type Scenedesmus to hydrogen over a number of hours enables it to carry out anaerobic photoreduction.

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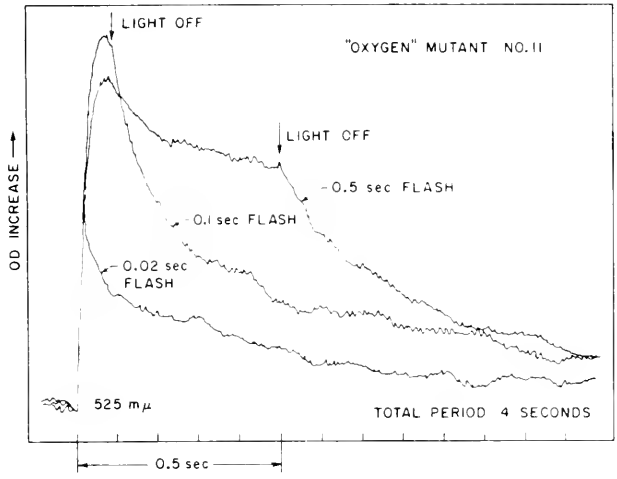

Fig. 7 Absorption changes in Mutant 11 with flashes of different duration. Each curve is the summation of 50 repetitions of the 4 second light-dark cycle.

De-adaptation at high light intensities is prevented by the addition of 3(3,4-dichlorophenyl)-1-1-dimethylurea (DCMU) to prevent the evolution of oxygen. Scenedesmus thus treated behaves very much like untreated Mutant 11 in its capacity for photoreduction(9); it was therefore not unexpected that the adapted wild type should display the 525 mμ absorption shift with kinetics resembling those of Mutant 11, as can be seen in Fig. 8.

We are still not able to equate any of the spectral shifts in the region we have been examining, i.e. that between 380 mμ and 580 mμ, with either of the typical free radical signals. The presence or absence of characteristic EPR signals may in general be correlated with the photochemical behavior of the mutant; however, the rapid kinetics of the formation and decay of these signals in the mutants has not yet been studied. It is notable that all the major absorption changes appeared in each strain, whether or not metabolic activity was occurring. The kinetics of these changes were strikingly altered in the mutant cultures, and it is hoped that further study will provide a basis for explanation of these time course differences, and perhaps yet reveal underlying relationships between observations by these two differing experimental techniques.

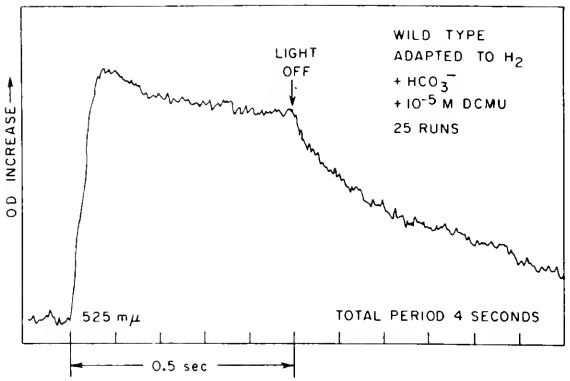

Fig. 8 Absorption change in hydrogen adapted wild type Scenedesmus with 0.1 mg $NaHCO_3$ in 10 ml of cell suspension and 10^{-5} M DCMU. The time course is similar to that of the "O_2" mutant, No. 11. The physiological behavior of these two preparations has been shown to be similar(9).

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A METHOD FOR CALCULATING QUANTUM YIELDS FOR THE FORMATION OF REACTION INTERMEDIATES

Daniel Rubinstein

The appearance or disappearance of absorption bands during illumination of photosynthetic organisms has been observed by many investigators beginning with Duysens (1). Whether or not these absorptions bands are due to compounds in the main path of photosynthesis could be best determined by quantum yield measurements. Photosynthesis in monochromatic red light has been observed to have a quantum requirement of eight (±2) quanta per molecule of oxygen evolved (2). A much larger quantum requirement for the appearance of an absorption band would suggest that this band is not due to a compound in the mainstream of photosynthesis.

Quantum yields have usually been estimated by examining the rate of increase in absorbance, and dividing this rate by that of the light absorption. The increase in absorbance is then converted into increase in concentration (moles per liter) by assuming a plausible value for the extinction coefficient. In some cases, such as the appearance of a band at 420 mμ in Porphyridium(3) or Chromatium (4) attributable to a cytochrome, the extinction coefficient is known from measurements in vitro; but for nearly all other difference bands, whose molecular origin is unknown, a guess must be made. In such cases, it is usually assumed that the extinction coefficient of the unknown compound is approximately the same as that of chlorophyll (or a similar organic pigment).

Many plots of absorption changes as a function of exciting light intensity have been published (4-10). In general, these have been plotted to determine whether several absorption bands belong to the same pigment, or whether an absorption change at a certain wavelength should be attributed to more than one pigment(6). If the plots for changes at different wavelengths are identical, they may be due to the same pigment. If a "light curve" (as these plots are designated) appears to have inflections, more than one pigment may be involved. Usually, no further information is sought from these plots. A more detailed analysis shows, however that additional information may be deduced from them. Specifically, the shape of the light curve may

permit evaluation of the quantum yield without having to assume an arbitrary value for the extinction coefficient, or without having to know exactly the amount of energy absorbed by the sensitizing pigment.

This evaluation is based on the following postulated reaction scheme:

$$\text{Chl} \underset{k_1}{\overset{KI_0}{\rightleftharpoons}} \text{Chl}^* \qquad (1)$$

$$\text{Chl}^* + A \xrightarrow{k_2} A^1 + \text{Chl} \qquad (2)$$

$$A^1 + B \xrightarrow{k_3} B^1 + A \qquad (3)$$

The first equation describes the absorption of light and of the pigment, Chl (which may be chlorophyll) to Chl*. The rate of formation of Chl* is equal to the number of absorbed quanta, KI_o. The rate constant, k_1, for the decay of Chl* includes all Chl* $\longrightarrow$ Chl transitions, except the one leading to the formation of the absorption band belonging to the molecule A^1 (eq. 2). In the second equation, it is postulated that the excited molecule, Chl*, reacts with A to form A^1. In the third equation, A^1 decays, during both light and dark periods, by reacting with a substrate, B, assumed to be present in excess.

The rate constant for the decay of A^1 (k_3, eq. 3) can be taken directly from the time-trace after cessation of illumination. The steady concentration of Chl* is easily seen to be

$$[\text{Chl}^*] = \frac{KI_o}{k_1 + k_2[A]}. \qquad (4)$$

The net rate of formation of A^1 during illumination is

$$\frac{d[A^1]}{dt} = k_2[\text{Chl}^*][A] - k_3[A^1]. \qquad (5)$$

It is further postulated that the saturation of the absorption change observed at high light intensities, is due to a limited quantity of reactant A, available for reaction with Chl*.

It follows that

$$[A] + [A^1] = [A^1]_{max} = [A_o] \tag{6}$$

By combining equations (5) and (6):

$$\frac{d[A^1]}{dt} = \frac{k_2 K I_o ([A^1]_{max} - [A^1])}{k_1 + k_2 ([A^1]_{max} - [A^1])} - k_3 [A^1] \tag{7}$$

The quantum requirement is the number of quanta needed for the appearance of an absorption band corresponding to a single molecule A^1. Eq. (7) predicts that the initial rate of formation of A^1 will be proportional to the absorbed light intensity. The ratio $\frac{d[A^1]}{dt} / KI_o$ when $[A^1] \longrightarrow 0$ has been defined as the quantum yield γ by Olson and Chance (4) and others (3)(11). In the above equation

$$\gamma = \frac{\frac{d[A^1]}{dt}}{KI_o} = \frac{k_2 K [A^1]_{max}}{k_1 + k_2 [A^1]_{max}} \tag{8}$$

$$A^1 \longrightarrow 0$$

The steady state expression following from (7) is

$$\frac{I_o}{[A^1]} = \frac{k_1 k_3}{k_2 K ([A^1]_{max} - [A^1])} + \frac{k_3}{K} \tag{9}$$

Only absorption changes, not concentration changes are actually observed; concentrations can be converted to absorbances (optical densities) by dividing them by ϵ (since $OD = \epsilon [A^1]$). Rearranging the terms, we obtain:

$$\frac{I_o}{OD} = \frac{k_3}{\epsilon K} + \frac{k_1 k_3}{k_2 K (OD_{max} - OD)} \tag{10}$$

This is the equation for a straight line when I_o / OD is plotted against $1/(OD_{max} - OD)$

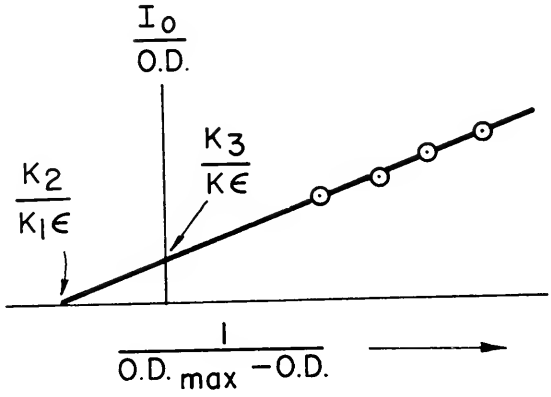

Figure 1

The intercept on the abscissa and the ordinate are respectively:

$$\frac{k_2}{k_1 \epsilon} \tag{11}$$

and

$$\frac{k_3}{\epsilon K} \tag{12}$$

The intercept on the abscissa is related to the quantum requirement $1/\gamma$ by the following expression:

$$\left(\frac{1}{\gamma} - 1\right) OD_{max} = \frac{k_1 \epsilon}{k_2} \tag{13}$$

The knowledge of the absolute number of absorbed quanta, $K I_o$ is not needed, to calculate the quantum requirement, since the constant, K, is absent from equation (13). The only terms needed for this determination are the change in OD reached at high light intensity. Calculation of the extinction coefficient from the intercept on the ordinate (fig. 1) requires, on the other hand, the knowledge of the number of absorbed light quanta, since the constant, K, remains in the expression (12).

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Discussion

In utilizing eq. (10) for the determination of quantum yields and extinction coefficients by extrapolation of the experimental curves to the two axes, accurate knowledge of several variables is required. A "close fit" of the individual points to the straight line is essential for the extrapolation to be meaningful. It is essential that the relative value of I be measured by means of a linear detector, such as a photomultiplier, or by utilizing a constant source of light with calibrated neutral density filters.

In this determination of quantum yield, we assume a particular set of reactions in which k_1, k_2 and k_3 were assumed constant and independent of I (eq. 1-3). Also, the conservation law was assumed to apply to A; that is $A + A^1$ was assumed to be constant. This idealized case may not always be realized, especially at high light intensities. This will be indicated by deviations from the straight line in our plot. For example, a positive deviation might mean that k_1 increases with light intensity. Other deviations in the same direction may be caused by increases in the rate of eq. (3) due to a photochemical reaction supplying substrate for the back reaction. These and other deviations can often be revealed by other plots such as those of absorbance vs. time or temperature.

Any intermediate A^1 which is present in limiting concentration could be detected not only by the absorption of light, but by other methods (eg. electron spin resonance) and the same equation will apply, provided the effect reaches saturation by the same reaction mechanisms. It is to be noted that the plot in fig. 1 does not give the "total" number of absorbed quanta needed to produce one molecule of A^1, but that of the quanta absorbed by the "active" pigment participating in eq. (1). The quantum yields calculated from the above plot may be used in the study of light reaction mechanisms in mixtures of "active" and "inactive" pigments, particularly when the "total" quantum yield can be obtained by conventional methods.

A more detailed study of these relationships will be published later. Thanks are due to Professor Eugene Rabinowitch, Professor Gregorio Weber, Professor Bernard Abbott and Dr. Ashish Ghosh for valuable discussions.

Daniel Rubinstein

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LIGHT-INDUCED RAPID ABSORPTION CHANGES DURING PHOTOSYNTHESIS. II. 430 mμ ABSORPTION CHANGES IN AGED CHLOROPLASTS IN THE PRESENCE OF PMS AND ASCORBATE

Bacon Ke

Chemical separation of two light reactions in photosynthesis and the reconstitution of the reaction system with artificial reagents have been amply demonstrated. Vernon and Zaugg [1] have shown that aged chloroplasts which had lost the capacity for oxygen evolution were able to photoreduce TPN when reduced DPIP was added. Other evidence for the chemical separation of two light reactions has been obtained from experiments on the relief of CMU inhibition of TPN reduction by ascorbate and redox dyes [2] and from experiments with mutants of Chlamydomonas reinhardi [3] and Scenedesmus [4].

Some preliminary observations on the 430 mμ absorption changes in aged chloroplasts and the response of these absorption changes to ascorbate and PMS will be reported in this note. In aged chloroplasts the 515 mμ absorption increase was either negligible or completely absent, indicating that the reaction associated with oxygen evolution was inactivated.

Rapid absorption changes were studied with an instrument basically similar to that reported by Witt [5]. Construction details of the instrument used in the present work will be reported elsewhere [6]. Polychromatic red (620 - 720 mμ) flashes with a duration of 2×10^{-5} sec were used for excitation. Aging of spinach chloroplast was done at 4° C for one week. The chloroplast suspension usually contained about 30 μg chlorophyll in a total volume of 3.0 ml 0.1 M pH 7 phosphate buffer.

Whereas fresh chloroplasts do not show any absorption change at 430 mμ, at least not under the experimental conditions and sensitivity used here, aged chloroplasts show a light-induced absorption decrease (Fig. 1, curve a). At the time resolution available, the rise time of the signal was estimated to be less than 10^{-4} sec, and the half life of decay was $4 - 5 \times 10^{-2}$ sec.

The 430 mμ absorption change in aged chloroplasts can be completely abolished by 2×10^{-5} M ferricyanide, but is unaffected by ferrocyanide at a concentration as high as 10^{-4} M. In a reducing medium of 2×10^{-3} M ascorbate, the absorption change was enhanced and the half life was shortened to 2×10^{-2} sec (Fig. 1, curve b). The loss of 430 mμ absorption change caused by ferricyanide can be fully restored by adding ascorbate.

Upon addition of PMS to a concentration of 3×10^{-6} M to the aged

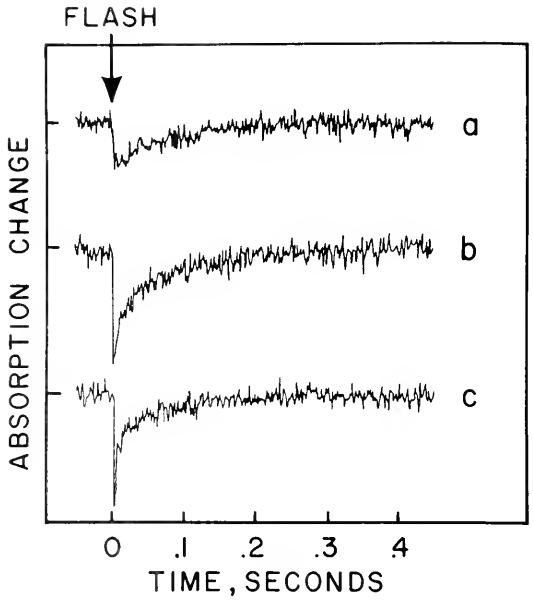

Fig. 1. Light-induced absorption change at 430 mμ in:
a. week-old chloroplasts.
b. a + 2 x 10^{-3} M ascorbate.
c. b + 3 x 10^{-6} $\underline{M}$ PMS.

chloroplasts already containing ascorbate, the absorption change was immediately converted to one composed of a rapidly decaying portion ($\sim 10^{-3}$ sec) superimposed on a portion having the same decay time as before PMS addition (Fig. 1, curve c).

By varying the measuring-beam wavelength between 400 and 450 mμ, a difference spectrum was obtained as shown in Fig. 2. The spectrum shows a broad negative peak covering the 420-430 mμ region and a smaller positive peak in the 405 mμ region.

Examination at 703 mμ revealed an absorption decrease with kinetic behavior very similar to that of the slow portion of the 430 mμ change. No rapidly decaying portion was observed in the 703 mμ absorption change.

The composite absorption change shows an interesting dependency on the intensity of the excitation flashes (Fig. 3). Starting from a given saturating intensity (I_{max}) and gradually decreasing it, the signal remained practically unchanged down to 50% intensity. Decreasing the intensity to below 50%, the rapidly decaying portion started to disappear. Between 30 and 15% intensity,

only the slowly decaying portion remained. Below 15% intensity, the slowly decaying portion also decreased.

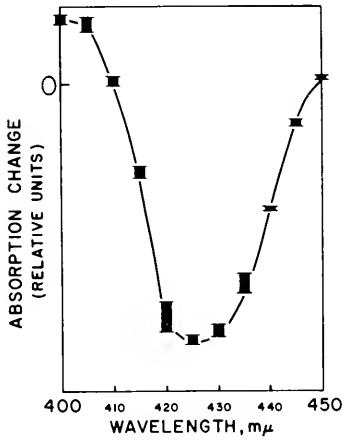

Fig. 2. Difference spectrum of aged chloroplasts in the presence of 2×10^{-3} M ascorbate and 3×10^{-6} M PMS.

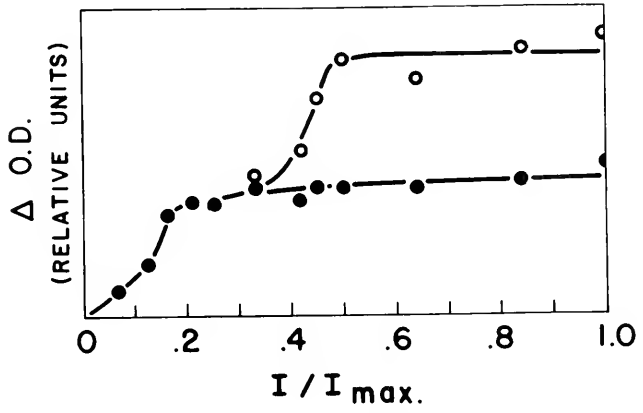

Fig. 3. Dependency of the composite peak on illumination intensity.
o total peak height.
• height of the slowly decaying portion.

Because the 430 mμ absorption change in aged chloroplasts was enhanced and its decay was accelerated by ascorbate, and because the absorption change was abolished by ferricyanide, it may be inferred that an oxidation reaction is responsible for the absorption decrease. Since the absorption decrease occurred at both 430 and 703 mμ, and the slowly decaying portion of the 430 mμ absorption change and the 703 mμ absorption change have the same kinetics, it is reasonable to assume that these absorption changes are caused by the oxidation of chlorophyll, probably the far-red absorbing pigment P-700 [7].

The breadth and peak position of the difference spectrum in Fig. 2 indicate that cytochrome oxidation, probably that of cytochrome f, may also be partly responsible for the 430 mμ absorption change. Thus, the initial absorption decrease may be interpreted as being due to photooxidation of chlorophyll, part of which rapidly extracts electrons from cytochrome f in the presence of ascorbate and PMS at the stated concentrations. The light intensity dependency of the composite curve suggests that the latter reaction occurs only when the light intensity exceeds a certain level. Cytochrome f then rereduces rapidly in the presence of PMS and chlorophyll re-reduces more slowly (by reduced cytochrome, PMS, ascorbate, or endogenous reductants). A similar reaction route has recently been proposed by Witt and co-workers for PMS concentrations less than 10^{-5} M, but no experimental details were given [8].

Absorption decreases at 430 mμ with rapid decay times of $\leq 10^{-4}$ sec have previously been reported by Moraw and Witt [9]. The so-called "type O" change has been attributed to the $\pi - \pi^*$ triplet state. The so-called "type 1" absorption decrease has been observed in many types of algae containing chlorophyll a and consequently described as due to the formation of a chlorophyll a derivative. The "type 1" signal was observed only at high illumination intensities and no saturation could be reached even at extremely high intensity. Judging from these characteristics, it can be concluded that the 430 mμ signal observed in the present work is not identical with either the "type O" or "type 1" 430 mμ signals reported. Furthermore, the difference spectra for the various types of absorption changes are entirely different.

In CMU-treated chloroplasts with ascorbate and PMS at concentrations similar to those used here, Jagendorf and Margulies concluded from a high ATP/TPNH ratio that a cyclic electron flow must also have occurred in addition to photoreduction of TPN. It is not known whether a similar situation exists here, and if so, what effect it might have on the transient absorption change. However, experiments on the light-induced 430 mμ absorption changes in the presence of ascorbate and PMS were conducted under both aerobic and anaerobic conditions, and practically identical results were obtained. Under anaerobic conditions PMS should exist exclusively in the reduced state in the presence of excess ascorbate. More extended and detailed experiments on these absorption changes will be reported elsewhere.

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II. ELECTRON TRANSPORT PATHS - BIOCHEMICAL INVESTIGATIONS

THE ELECTRON TRANSPORT SYSTEM OF PHOTOSYNTHESIS DEDUCED FROM EXPERIMENTS WITH MUTANTS OF CHLAMYDOMONAS REINHARDI

R. P. Levine

INTRODUCTION

Six years have elapsed since Emerson and his coworkers[1] described two different types of light effects in green plant photosynthesis, and it has been only three years since Hill and Bendall suggested that the electron transport system of photosynthesis could be interpreted in terms of two distinct light-dependent reactions coupled by at least one light-independent reaction[2]. Subsequently, there has been almost a surfeit of publications on the two light effects in photosynthesis; merely a brief reference to the papers presented in this and other recent symposia is sufficient to emphasize the impact that Emerson's original contribution has had on contemporary research into the mechanism of photosynthesis[3-5].

It is indeed noteworthy that at present there is a fair extent of uniformity among the several schemes proposed for the electron transport system of photosynthesis. However, the general nature of the schemes, and the lack of sufficient data for their support, makes it virtually impossible to accept or deny any one of them. The uniformity of most of the popular schemes is a mixed blessing, for it suggests on the one hand that diverse experimental approaches are leading to a set of final and general conclusions. On the other hand, the specific and significant details by which these schemes differ suggest that there is much to learn before we have a complete understanding of the mechanism of photosynthesis.

The purpose of this contribution to the symposium is to present data concerning reactions in the electron transport system of photosynthesis of the unicellular green alga, Chlamydomonas reinhardi, as studied with the wild type strain and mutant strains that are unable to carry out normal photosynthesis. Furthermore, these data will be used as the basis of a model for a sequence of reactions in the electron transport system.

Three years ago we became interested in the genetic control of photosynthesis. The investigation, however, soon turned from one having an orientation primarily of a genetic sort to one more directly concerned with the mechanism of photosynthesis per se. The work was begun with the aid of a mutant strain of C. reinhardi that was unable to fix carbon dioxide by photosynthesis[6]. It was assumed that this, and other mutant strains isolated subsequently[7, 8],

were unable to carry out normal photosynthesis because of gene mutation that either;

1) affected the synthesis or activity of enzymes responsible for the formation of components in the electron transport system of photosynthesis; or,

2) affected the synthesis or activity of enzymes whose function lies in synthetic processes attending normal chloroplast development; or,

3) affected the synthesis or activity of enzymes responsible for the formation of components of the reductive pentose cycle.

Clearly, the actual nature of the genetic block could be any one of the aforementioned and result in a cell's inability to carry out normal photosynthesis. Furthermore, it was realized that it might be difficult to distinguish, at least on a phenotypic basis, between certain classes of mutants in view of the intimate relationship between chloroplast structure and function.

The results and conclusions presented here derive principally from biochemical experiments utilizing chloroplast fragments and, as such, they may not necessarily reveal what happens in the intact cell or chloroplast. However, the model presented here is in substantial agreement with some of the models that have been proposed from studies with whole cells or whole chloroplasts[3-5].

METHODS

The methods we have used for investigating the electron transport system of photosynthesis in C. reinhardi stem largely from the well established techniques used with higher plants. A description of the methods for assaying the activity of enzymes related to photosynthesis, as well as the techniques for investigating photosynthetic reactions by whole cells and isolated chloroplast fragments of C. reinhardi have been published (see principally references 9-11). Details for the procedures used in isolating and measuring the quantity of different components of the electron transport system of photosynthesis have also been described[12, 13]. The methods for investigating the electron spin resonance signals in C. reinhardi have been described elsewhere[14]. Photoreduction[15] by whole cells following adaptation to hydrogen gas was measured manometrically as consumption of hydrogen in the light or as carbon dioxide fixation in the light from C^{14}-labeled bicarbonate. The procedures, details of which will be published elsewhere[16], are similar to those used for Scenedesmus[17].

GENERAL DESCRIPTION OF THE MUTANT STRAINS

The mutant strains of C. reinhardi used for the experiments reported here were derived from the wild type strain (No. 137c) by induction with

ultra-violet light, followed by screening for their inability to fix carbon dioxide in the light[7]. Unlike the wild type strain, each of the mutant strains will not grow in minimal medium unless it is supplemented with sodium acetate[18].

Four different mutant strains will be considered here; namely, ac-21, ac-115, ac-141, and ac-208 (the symbol ac refers to acetate dependence). With the exception of ac-141 and ac-208, the mutants lie in different linkage groups. Although ac-141 and ac-208 are linked they lie about 10 map units apart on opposite sides of the centromere in linkage group III (see references 19 and 20 for details of the techniques of genetic analysis). In terms of current genetic theory, each mutation should be located in a different cistron or functional unit. That is, each mutation most likely represents a genetic alteration that affects the synthesis or activity of a different enzyme.

These mutant strains, while unable to fix carbon dioxide by photosynthesis at the wild type rate, resemble wild type in two important characteristics. First, electron microscopy has revealed that ac-21, ac-115 and ac-141 have normal chloroplast structure[21]; ac-208 has not been examined. Second, there are no major differences in chlorophyll content[12]. There are, however, minor differences in carotenoid content, but these may be trivial in so far as their being causally related to the inability of the mutant strains to carry out normal photosynthesis[13].

REACTIONS OF THE ELECTRON TRANSPORT SYSTEM OF PHOTOSYNTHESIS

The rationale of the experiments with the mutant strains of C. reinhardi is simply one of inquiring into which known reactions of photosynthesis whole cells or isolated chloroplast fragments can or cannot perform, and then attempting to reconstruct the sequence of reactions which best fits the observed results. Thus, we are following, by analogy, one of the classical procedures of biochemical genetics to determine the sequence of events as they occur in a particular biosynthetic pathway.

Except for recently obtained data pertaining to the mutant strain ac-208, and to both photoreduction and photophosphorylation as they relate to wild type and the four mutant strains, the results summarized here are discussed in a series of publications[6, 8-11, 14].

Carbon dioxide fixation in whole cells by photosynthesis and photoreduction

It has been shown that, in comparison to the wild type strain, whole cells of the mutant strains are highly deficient in their ability to fix carbon dioxide by photosynthesis[8]. The maximum rate of fixation by a mutant strain (ac-21) was about two per cent of the wild type rate.

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Wild type cells fix carbon dioxide by photoreduction after being adapted to hydrogen in the dark for a period of 15 minutes. The maximum rate of photoreduction was obtained at a light intensity of about 1500 lux. At intensities greater than 2000 lux the cells reverted to photosynthesis. However, photoreduction was obtained at a light intensity of 10,000 lux in the presence of 1 x 10^{-5}M DCMU. At this concentration DCMU causes a 99 per cent inhibition of photosynthetic oxygen evolution and carbon dioxide fixation.

At 1500 lux the mutant strains ac-115 and ac-141 gave rates of carbon dioxide fixation and hydrogen consumption comparable to the wild type rate, whereas the rates of ac-21 and ac-208 were negligible. The addition of DCMU had little effect upon photoreduction by ac-115 and ac-141, and photoreduction was obtained at 10,000 lux in the absence of DCMU.

Both ac-115 and ac-141 resemble a mutant strain of Scenedesmus described by Bishop[17] that can carry out photoreduction but does not evolve oxygen by photosynthesis and has no Hill reaction with p-benzoquinone.

The Hill reaction

Further differences between the mutant strains were revealed in a study of the Hill reaction both by whole cells and chloroplast fragments[8-10]. As seen in Table I, ac-115 and ac-141 have no Hill reaction with a variety of Hill oxidants; ac-21 and ac-208 show Hill reaction activity, except that the latter does not have activity when the oxidant is potassium ferricyanide.

Table I

Hill reaction, TPN photoreduction, and photophosphorylation by wild type and mutant strains of C. reinhardi

	Hill reaction*			TPN reduction**		Photophosphorylation***		
Strain	DPIP	Cyt c	FeCy	a‡	b	PMS	Vitamin K_3 & FMN	DPIP & Ascorbate
wild type	55.2	28.8	324	35.4	7.8	124	50	8
ac-21	54.6	6.6	114	0	9.0	0	0	0
ac-115	0	0	0	0	13.8	112	30	-
ac-141	0	0	0	0	22.2	209	43	-
ac-208	53.2	14.7	0	0	0	0	0	-

* μmoles Hill oxidant reduced/hr/mg chlorophyll

** μmoles TPNH/hr/mg chlorophyll

*** μmoles Pi esterified/hr/mg chlorophyll

‡ a signifies TPN reduction when the source of electrons is from water and b signified TPN reduction when the source of electrons is from DPIP and ascorbate

TPN photoreduction

An investigation of TPN photoreduction showed that all of the mutant strains possessed an active PPNR and pyridine nucleotide transhydrogenase[10]. However, as shown in column five of Table I, chloroplast fragments from each of the mutant strains were ineffective in the photoreduction of TPN in the presence of an excess of purified PPNR when the source of electrons was water[9, 10]. On the other hand, all strains except ac-208 were capable of TPN photoreduction when the electron donor was a catalytic amount of DPIP in the presence of a substrate concentration of ascorbate[9, 19].

Photophosphorylation

Recent investigations have revealed that wild type, ac-115, and ac-141 are capable of carrying out cyclic photophosphorylation with PMS, or the combination of vitamin K_3 and FMN, as the added electron carriers whereas ac-21 and ac-208 are not[22]. Since ac-21 can photoreduce TPN from DPIP and ascorbate, it was considered important to determine whether or not TPN photoreduction could be accompanied by photophosphorylation in this strain. The wild type strain can carry out non-cyclic photophosphorylation linked to TPN reduction in this manner (Table I). Numerous attempts with ac-21, however, have failed to give non-cyclic photophosphorylation, though in each instance there was the expected rate of TPN reduction.

Electron spin resonance

Results of an investigation of electron spin resonance[14] have shown that wild type, ac-21 and ac-208 have the two ESR signals (I, the fast, narrow signal and II, the slow broad signal) that are characteristic of Chlorella, Chlamydomonas, and Scenedesmus[23-25]. However, signal II is missing in ac-115 and ac-141. Signal I in C. reinhardi is light-dependent, and has an action spectrum that resembles the absorption spectrum of chlorophyll a. Signal II occurs in the absence of light, at least in cells that have been cultured in the light[14].

COMPONENTS OF THE ELECTRON TRANSPORT SYSTEM OF PHOTOSYNTHESIS

The inability of any one of the mutant strains to carry out normal photosynthesis might be accounted for on the basis of some change in either the quantity or chemical nature of one of the possible components of the electron transport system. We have analyzed, in part, the cytochrome b_6, cytochrome f, plastocyanin, plastoquinone, and carotenoid content of wild type, ac-21, ac-115, and ac-141[9, 12, 13, 26]. Since we are still engaged in these analyses, the data presented here are incomplete.

Table II summarizes data regarding cytochrome b_6, cytochrome f, and plastocyanin[12]. It can be seen that there is approximately three to four times as much cytochrome f in ac-115 and ac-141 as compared to wild type and ac-21. The cytochrome b_6 as well as the plastocyanin content of the strains is about equal.

Table II

Cytochrome b_6, cytochrome f, and plastocyanin content of wild type and mutant strains of C. reinhardi

Strain	Cytochrome content		Plastocyanin content
	Cytochrome b_6	Cytochrome f	
	moles chlorophyll/mole cytochrome		moles chlorophyll/g atom Cu
wild type	90	363	500
ac-21	113	489	530
ac-115	154	174	429
ac-141	100	167	560

An analysis of the carotenoid pigments in wild type, ac-21, ac-115, and ac-141 has revealed similarities among the mutant strains which distinguish them from the wild type strain when they are cultured in the light[13]. Each mutant strain has both a lower carotenoid content and a lower beta-carotene/alpha-carotene ratio than light-grown wild type. Interestingly, both the lower total carotenoid content and the lower beta-carotene/alpha-carotene ratio are characteristic of wild type when it is cultured in the dark. However, in spite of this similarity between the mutant strains and dark-grown wild type, each mutant strain has a pattern of types and amounts of carotenoids that distinguishes it from the other mutant strains and from both light- and dark-grown wild type.

The initial investigation of plastoquinone in wild type, ac-21, ac-115, and ac-141 revealed that both ac-115 and ac-141 have five-fold less plastoquinone than wild type and ac-21[9, 12]. This investigation preceded the important discovery of Crane and his coworkers in which it was demonstrated that several different plasto- and tocopherylquinones could be extracted from spinach chloroplasts[27, 29]. Accordingly, our original data for wild type and the mutant strains (Table III) were representative of a combination of quinones, and most likely some of the quinones were missing because of the extraction procedure used. We have recently undertaken a more extensive analysis of the quinones of C. reinhardi, and it has revealed a variety of both plasto- and tocopherylquinones similar to those found in spinach.

Table III

Plastoquinone content of wild type and mutant strains of C. reinhardi

Strain	Moles chlorophyll/mole plastoquinone
wild type	15
ac-21	30
ac-115	99
ac-141	74

DISCUSSION

Each of the four mutant strains of C. reinhardi under consideration is unable to carry out normal photosynthesis because some portion of the electron transport system does not function. This loss of function results from the mutation of either a structural or regulatory gene. The expression of this mutation is seen experimentally as a block in photosynthesis, and the term block will be used to describe the point or points at which the electron transport system is stopped. It is assumed that in each mutant strain the genetic change affects an enzyme that is at least indirectly concerned with the electron transport system. The term block, however, is not meant to imply a knowledge of the specific enzymes involved. Furthermore, a single mutation could result in a block at more than one point. If, for example, two components of the system are formed as part of the same biosynthetic pathway, a mutation that affects some common, early step in their biosynthesis could result in the loss of both components and, consequently, the system would be blocked at two different points. In addition, the loss of some single component might result not only in a block within the electron transport system but in the coupling of photophosphorylation to the system as well.

The initial model proposed for the electron transport system of photosynthesis in C. reinhardi[9] was based upon the hypothesis of Hill and Bendall[2] that there are two different, light-dependent reactions coupled by at least one light-independent, exergonic reaction. According to this model, and using the terminology of Duysens, light absorbed by system II results in the oxidation of water coupled to the formation of a photoreductant; light absorbed by system I results in the formation of a photo-oxidant and the reduction of TPN. Overall, the photoreductant produced in system II is oxidized by the photo-oxidant produced in system I.

The model to be discussed here (Fig. 1) retains the general features of the one presented earlier[9]. It has gained additional support from recent studies of the mutant strains of C. reinhardi. As further experiments are performed, and other mutant strains studied, the details of the model may change. This is, therefore, only a working model, but it most nearly

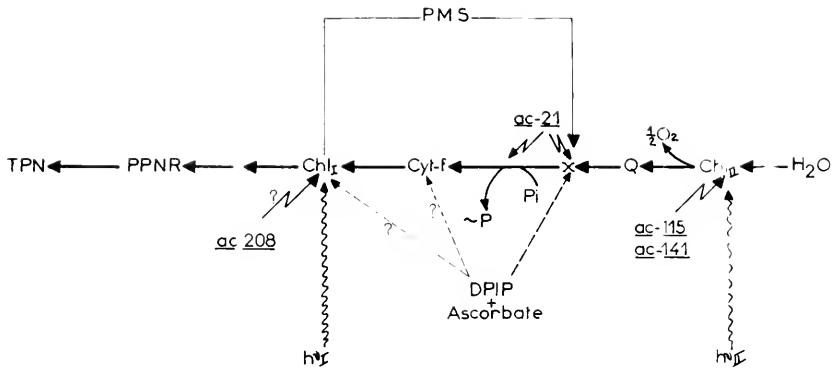

Figure 1. A Model of the Electron Transport System of Photosynthesis in C. reinhardi as Deduced from an Investigation of Four Mutant Strains. (See text for explanation)

accommodates the facts we have obtained so far, and we believe it contains the least number of assumptions. Quite clearly, the model is not unique, for it draws heavily upon findings of other investigators whose approach has been somewhat different from ours.

Two light-dependent reactions and at least one light-independent reaction

Evidence for the two different light-dependent reactions of C. reinhardi shown in the model comes from results of experiments with all four mutant strains. All of the data obtained for ac-115 and ac-141[9, 10] are consistent with the hypothesis that these two strains are blocked in a reaction associated with system II, in which a photoreductant is produced coupled with the oxidation of water. Chloroplast fragments obtained from these strains showed no Hill reaction activity and were unable to photoreduce TPN. It was predicted that if the block were only in system II, chloroplast fragments could carry out the photoreduction of TPN if a reductant were supplied implying that system I was able to function in these two strains. This prediction was borne out when it was shown that chloroplast fragments of ac-115 and ac-141 could photoreduce TPN from DPIP and ascorbate. In this respect both strains resemble wild type which has been inhibited with DCMU or o-phenanthroline, for under these conditions wild type chloroplast fragments can photoreduce TPN in the presence of DPIP and ascorbate.

Further confirmation of the ability of these two mutant strains to carry out part of the photosynthetic electron transport was obtained when it was established that both were able to do cyclic photophosphorylation with PMS and to fix carbon dioxide by photoreduction.

It was also demonstrated that both ac-115 and ac-141 had retained the sharp, fast ESR signal but that the slow, broad signal was missing. The former signal has been attributed to a long wave length form of chlorophyll a such as P-700[25, 30], whereas the latter signal may be associated with chlorophyll b, for this signal is absent in a chlorophyll b-less mutant of Chlorella[23]. In the case of ac-115 and ac-141 it is tempting to correlate the absence of this signal with the inability of these mutant strains to carry out a reaction in system II.

In contrast to both ac-115 and ac-141, ac-208 has Hill reaction activity with all of the Hill oxidants tested except ferricyanide, and yet it cannot photoreduce TPN from DPIP and ascorbate. These results suggest that the block in ac-208 lies at a side in the system subsequent to the point of entry of electrons from DPIP and ascorbate. The block could lie in system I. However, there is no direct evidence for this, and ac-208 has both ESR signals.

The two light-dependent reactions occur in ac-21. However, they must be coupled by at least one light-independent reaction which is blocked in this mutant strain. A photoreductant is produced by system II in ac-21 as evidenced by the fact that there is Hill reaction activity. However, this photoreductant apparently cannot be utilized, for DPIP and ascorbate must be provided in order to obtain TPN photoreduction. These results can be best explained by assuming that a block lies in a light-independent reaction between systems I and II. This explanation is supported by the observation that both ESR signals are generated in cells of ac-21. Thus, inasmuch as the two different ESR signals may reflect systems I and II, the mutant strain is identical to wild type.

Both ac-21 and ac-208 pose some interesting questions regarding the site of action of ferricyanide into the electron transport system of C. reinhardi. Witt, Müller, and Rumberg[31] suggest that ferricyanide belongs to a group of oxidants, termed ox S_I, whose reduction is associated with the oxidation of Chl_I. Accordingly, any block in the electron transport system of C. reinhardi lying at a site after system II should result in the absence of a Hill reaction with ferricyanide. Thus, since ac-21 and ac-208 are blocked after system II, neither of them should have a Hill reaction with ferricyanide. This is contrary to our observations, for ac-21 does have Hill reaction activity with this oxidant, albeit at a rate that is about three times lower than that of wild type. It is conceivable that the Hill reaction with ferricyanide could proceed via system II alone in ac-21. If this assumption is made, however, it would be expected that ac-208 would also give a Hill reaction with ferricyanide. Of course, the dilemma with ac-208 could be avoided by making the second assumption that there are two blocks in ac-208; namely, that there is a block in electron transport between the point of entry of electrons from DPIP and the reduction of TPN, and that ac-208 lacks a component of the electron transport system unique to the ferricyanide Hill reaction.

These ad hoc assumptions, however, should not be considered significant in the absence of experimental evidence. If the Hill reaction with ferricyanide in ac-21 proceeds from system II alone then its action spectrum might be different from that obtained with wild type. We have recently begun, in collaboration

with G. Gingras, measurements of the action spectrum of the ferricyanide Hill reaction by chloroplast fragments of both wild type and ac-21. These measurements, obtained with the aid of an oxygen electrode, have revealed so far that the action spectrum for wild type is very similar to the action spectrum for photosynthetic oxygen evolution by whole cells.

Photophosphorylation

Additional important information for the design of the model was obtained from an investigation of photophosphorylation by wild type and the four mutant strains.

Photophosphorylation has been studied in wild type in several different ways[11]. Cyclic photophosphorylation was obtained with either PMS or the combination of vitamin K_3 and FMN as electron carriers. Cyclic photophosphorylation with PPNR[32] has not been tested. Non-cyclic photophosphorylation coupled to the photoreduction of TPN was found to occur with either water or DPIP and ascorbate as the reductant. However, non-cyclic photophosphorylation coupled to ferricyanide reduction could not be demonstrated.

The ratio of one ATP produced per two electrons transferred during non-cyclic photophosphorylation in C. reinhardi suggests that there is only one site for non-cyclic photophosphorylation. Furthermore, in agreement with Losada, Whatley, and Arnon[33], non-cyclic phosphorylation lies at a point after the entry of electrons from DPIP and ascorbate into the electron transport system.

In the model for electron transport in C. reinhardi under consideration here, the site for photophosphorylation has been placed tentatively between X (a component of the electron transport system and the assumed point of entry of electrons from DPIP and ascorbate) and cytochrome f. A photophosphorylation at this site would, accordingly, be coupled to the oxidation of a reduced X and the reduction of an oxidized cytochrome f. Recently, Forti, Bertole, and Parisi[34] have shown that photophosphorylation in spinach chloroplasts can be coupled to the reduction of cytochrome f and that the stoichiometry is one ATP produced per two electrons transferred. It has also been shown that cytochrome f can be oxidized by system I[35-37]. Further, in Anacystis nidulans[35] cytochrome f can be reduced by system II.

Cyclic photophosphorylation with either PMS, or the combination of vitamin K_3 and FMN, was obtained only with the mutant strains ac-115 and ac-141. This indicates that the cyclic pathway enters the electron transport system after the block in these two strains and, accordingly, does not depend upon a photoreductant produced in system II. The fact that neither ac-21 nor ac-208 gave cyclic photophosphorylation supports the contention that these strains are blocked at sites after the point of entry of the cyclic pathway into the electron transport system.

Since ac-21 can photoreduce TPN from DPIP and ascorbate it was assumed that, like wild type, it would also carry out photophosphorylation with DPIP and ascorbate; thus, it would be possible to localize the block in this mutant strain to a position before the site of entry of electrons from DPIP and ascorbate. As mentioned earlier it has thus far been impossible to obtain photophosphorylation in this manner with ac-21 even though TPN is reduced.

There are several ways one may interpret the results obtained with ac-21. First, it might be assumed that there is a block between X and cytochrome f, and in order to obtain TPN photoreduction from DPIP and ascorbate, electrons from these donors must enter the system at a point after X and the site of phosphorylation. This might be at cytochrome f or at Chl_I. Second, the block might be in the formation of X itself; it may be either lacking, deficient, or inactive. Once again, in order to explain TPN photoreduction from DPIP and ascorbate, the electrons would have to enter at some alternate site. Third, X might be essential for both electron transport and for the coupling of phosphorylation to the electron transport system. Consequently, if X were lacking, deficient, or inactive in ac-21 both electron transport and phosphorylation would be blocked.

Photoreduction and cyclic photophosphorylation

A mechanism exists in C. reinhardi for the production of ATP independently from the oxygen evolving pathway of photosynthesis. Wild type cells can fix carbon dioxide by photoreduction in the presence of a concentration of DCMU that almost completely inhibits both photosynthetic oxygen evolution and carbon dioxide fixation. Photoreduction also occurs in cells of ac-115 and ac-141 where the oxygen evolving pathway is blocked as a consequence of mutation. Both of these mutant strains were also found to carry out cyclic photophosphorylation with PMS, thus confirming the finding of several investigators that photophosphorylation with PMS is independent of the oxygen evolving pathway.

These results suggest that ATP is generated during photoreduction by a process of cyclic photophosphorylation, and photoreduction in C. reinhardi, therefore, may be similar to bacterial photosynthesis. That is, a hydrogenase acts to reduce TPN by a light-independent reaction and ATP is produced via cyclic phosphorylation. However, the possibility that the hydrogenase may provide electrons for the production of ATP by a non-cyclic photophosphorylation cannot be excluded.

Cytochrome f and quinones

Among the possible components of the electron transport system in C. reinhardi, the quinones and cytochrome f are the only ones for which marked quantitative differences have been found[12]. The low level of plastoquinone in both ac-115 and ac-141 is of particular interest. The plasto- and tocopherylquinones have been implicated in photosynthesis because they are localized in

chloroplasts[38] and undergo reduction in chloroplasts in the light[39-41]. Furthermore, it has been shown that they are essential for the Hill reaction[27, 41-45], the photoreduction of TPN[46], and for cyclic photophosphorylation[47, 48]. Thus, it appears that different plasto- and tocopherylquinones may function at different sites in the electron transport system.

The low plastoquinone content of both ac-115 and ac-141 is not sufficient to account for the complete absence of a Hill reaction in these strains, at least on the basis of the results obtained by Bishop[42] for sugar beet chloroplasts. However, it is possible that these two mutant strains lack or are deficient in a specific plasto- or tocopherylquinone that functions at a site associated with system II and the oxygen evolving portion of the system, whereas the quinones that remain act at some different site or sites.

The cytochrome f content of both ac-115 and ac-141[12] is about three to four times greater than that of wild type and ac-21. Coupled with this increase there is at least a doubling of the rate of TPN reduction from DPIP and ascorbate (Table I), and also in cytochrome photo-oxidase activity[10]. These observations are consistent with the idea of Hill and Bendall[2] that cytochrome f is involved with system I, and with the observations of the light-dependent oxidation of cytochrome f in algae by system I[35-37].

CONCLUSIONS

Though our model for the electron transport system of photosynthesis in C. reinhardi lacks complete documentation, it most easily accommodates the observations that have been made with the wild type and four mutant strains. All of the data presented are consistent with a model for electron transport in which there are two light-dependent reactions separated by at least one light-independent reaction. The data suggest that there is a single site for non-cyclic photophosphorylation, and that cyclic photophosphorylation can occur independently of the oxygen evolving portion of the system. In addition, one or more plasto- or tocopherylquinones may play an integral role in the electron transport system.

The model presented here has the advantage of providing several predictions that can be tested experimentally. For example, if ac-21 lacks component X (Fig. 1), then it should be possible to detect the oxidation of cytochrome f by system 1 but not its reduction by system II. Further, both ac-115 and ac-141 may show the oxidation of cytochrome f in the light followed by its reduction in the dark[35]. In contrast, if the block in ac-208 lies in system I the mutant strain would be expected to show the reduction of cytochrome f by system II but not its oxidation by system I.

In addition to testing predictions such as these, the use of the mutant strains provides an opportunity to search for the function of different possible components of the electron transport system, for as mentioned at the outset of this discussion, mutations may have occurred at gene loci that affect the

biosynthesis of some of these components. The establishment of the loss or alteration of any one component in a given strain, taken in conjunction with what we have learned about the strain's loss of photosynthetic reactions, can provide strong evidence for the function of such a component.

Clearly, we have merely commenced an extensive series of investigations, for we have by no means exhausted all of the opportunities for different kinds of experiments with the four mutant strains, and we have yet to investigate in any detail some 15 additional mutant strains.

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EFFECTS OF QUINONES AND OXYGEN IN THE ELECTRON TRANSPORT SYSTEM OF CHLOROPLASTS

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Quinones always played a major role in new developments in extracellular photosynthesis with isolated chloroplasts. After Hill had demonstrated oxygen evolution accompanying the photosynthetic reduction of ferric salts by chloroplasts[1], Warburg introduced p-benzoquinone and naphthoquinone-sulfonate as Hill reagents[2]. In the discovery of photosynthetic phosphorylation by Arnon it became soon apparent that vitamin K_3 = methylnaphthoquinone is one of the most effective cofactors[3]. When the role of a new class of natural benzoquinones in oxidative phosphorylation of mitochondria (the ubiquinones) was investigated, Crane also found a similar, but somewhat different endogenous quinone in chloroplasts (plastoquinone)[4] and Bishop showed its importance in the photosynthetic reduction of ferricyanide by chloroplasts[5]. Quinones are therefore involved in the two principal photosynthetic reactions of chloroplast fragments—Hill reaction and photosynthetic phosphorylation—in two ways: as added substrate or cofactor and as an endogenous component of the electron transport chain.

1. Quinones as substrates for the Hill reaction

Numerous compounds have been tested and found suitable as Hill reagents. It does not seem surprising, that organic compounds, functioning as Hill reagents, have a quinoid structure, since this is the one most easily reduced. Aronoff and Wessels in 1952 investigated the photosynthetic reduction of a number of substituted benzo- and naphthoquinones by chloroplasts in relation to their redoxpotentials[6-7]. Wessels observed no reduction of compounds with a redoxpotential more negative than -100 mV. When Arnon had shown that the Hill reaction with ferricyanide or TPN was coupled to a stoichiometric ATP formation according to the equation[8]

$$X + H_2O + ADP + P_i \longrightarrow XH_2 + 1/2\ O_2 + ATP$$

it seemed desirable to investigate the behavior of benzoquinones again[9].

Table 1 shows the reduction of a number of substituted p-benzo-, naphtho- and anthraquinones in nitrogen by illumination with broken chloroplasts. Oxygen evolution as well as coupled ATP formation was measured.

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The table indicates, that in the presence of all quinones ATP is formed, which in most cases is accompanied by oxygen evolution. It seems safe to conclude, that substituted benzoquinones can be used as Hill reagents and that their reduction is coupled to ATP formation. The non-cyclic type of photophosphorylation[8] can be generalized to probably all Hill reagents.

However, as seen in table 1, stoichiometry between oxygen evolution and ATP formation is usually not obtained. With falling redoxpotential of the quinone, oxygen evolution is lagging behind ATP formation; when the redoxpotential is approaching zero V, no oxygen is evolved at all. This is in agreement with the results of Wessels[4]. The reason for this seems easy to understand. The more negative the redoxpotential, the more autoxidizable is the hydroquinone. Therefore the oxygen evolved in the reduction of the quinone is reacting back with the hydroquinone formed. This was already suggested by Wessels[4] as possible explanation for his failure to observe reduction of compounds with a redoxpotential below -1oo mV. That such backreactions occur, can now be seen by the formation of ATP without any measurable (by manometric techniques) oxygen evolution. The experiments in table 1 have been performed in nitrogen; apparently the small concentration of oxygen formed is sufficient to react with the hydroquinone formed. Therefore oxygen as well as the quinone/hydroquinone couple are then cycling.

substrate	µatoms O evolved	µmoles ATP formed	E'_o
---	o,1	o,3	
ferricyanide	5,o	4,8	
p-benzoquinone	5,8	2,8	293
toluquinone	4,4	3,o	237
2,3-dimethyl-p-benzoquinone	5,1	3,1	177
2,5-dimethyl-p-benzoquinone	3,9	3,9	176
trimethylbenzoquinone	4,1	5,1	1o2
2,6-dimethoxy-p-benzoquinone	3,3	5,4	53
2,6-dimethoxy-methylbenzoquinone	2,6	5,1	51
2-hydroxy-benzoquinone-5-propionate	o,1	2,5	27
2-methyl-naphthoquinone	o	7,4	-1o
phthiokol	o	6,1	-18o
anthraquinone-2-sulfonic acid	o	7,1	-25o

Table 1: Quinones as Hill reagents
Illumination for 15 min at 15° with 35ooo Lux in nitrogen. Each vessel contained in µmoles: trisbuffer p_H 8,o 8o; $MgCl_2$ 5; ADP 1o; P_i 1o; quinone 5 or ferricyanide 1o; and broken chloroplasts (P_{1s1}) with o,3 chlorophyll in a total volume of 3 ml.

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2. Quinones as cofactors of cyclic photophosphorylation

If the added components of the system are cycling, no substrate amounts of a quinone should be required. Indeed, all tested substituted o- and p-benzoquinones are cofactors of a cyclic photophosphorylation[9]: by illuminating chloroplasts with catalytic amounts of a quinone (or hydroquinone) ATP is formed (table 2 and 3). Since ATP formation with benzoquinones in catalytic amounts occurs only in the presence of oxygen[9], this has to be called an aerobic or pseudocyclic photophosphorylation.

o,1 µmol cofactor added	µmoles ATP formed
chlorogenic acid	8,2
caffeic acid	8,1
dihydroxyphenylalanin (DOPA)	7,8
dihydroxyphenylethylamin (Dopamin)	8,6
catechol	6,3
vitamin K_3	6,4

Table 2: o-Hydroquinones as cofactors of aerobic photophosphorylation (conditions as in table 1, 15 min light in air).

The term pseudocyclic was introduced by Arnon[10] to distinguish an oxygen dependent photophosphorylation from true oxygen independent cyclic photophosphorylation. In aerobic (pseudocyclic) photophosphorylation the cofactor is first reduced in a coupled Hill reaction. The reduced cofactor is then reoxidized by oxygen.

$$Q + H_2O + ADP + P_i \longrightarrow QH_2 + ATP + 1/2\ O_2$$

$$QH_2 + 1/2\ O_2 \longrightarrow Q + H_2O$$

sum:
$$ADP + P_i \longrightarrow ATP$$

The higher efficiency of certain cofactors of photophosphorylation in an atmosphere of air or oxygen has been observed[11-15]. All oxidizable compounds, whose oxidized form can act as Hill reagent have to be included in the list of cofactors of aerobic photophosphorylation, among them numerous o- and p- hydroquinones, some of which are mentioned in tables 2 and 3.

Substituted p- and o-benzoquinones, in catalytic amounts are not active as cofactors of true cyclic photophosphorylation in an atmosphere of nitrogen[9]. But a number of naphtho- and anthraquinones are effective in nitrogen[3,9]. Such ATP formation independent of oxygen was discovered by Arnon and later

o,1 μmol cofactor added	redoxpotential (E'_o in mV)	μmol ATP formed in N_2	air
p-benzoquinone	293	o,5	1,7
2,3-dimethoxy-p-benzoquinone	198	o,1	1,2
2,3-dimethyl-p-benzoquinone	177	o,4	1,3
2,5-benzoquinone-diacetic acid	167	o,1	4,7
2,3-dimethoxy-methylbenzoquinone	151	o,8	3,5
1,4-naphthoquinone-sulfonic acid	118	o,2	4,3
2,6-dimethoxy-benzoquinone	53	o,4	5,7
phenanthrenquinone	28	o,6	6,7
2-hydroxy-benzoquinone-propionate	27	o,1	4,9
vitamin K_3	-1o	2,o	4,7
2-hydroxy-naphthoquinone	-154	4,3	6,8
phthiokol	-18o	6,7	1o,o
anthraquinone-2-sulfonic acid	-25o	5,o	5,o

Table 3: Quinones as cofactors of photophosphorylation in nitrogen and in air (conditions as in table 1).

termed cyclic photophosphorylation[8], to distinguish it from phosphorylation accompanying non-cyclic electron flow in the Hill reaction, which was discussed above. However, Arnon has surmised that the phosphorylating step is identical in the two systems[18]. In the truly cyclic electron flow, the reduced cofactor cannot be reoxidized by oxygen (being absent), but is presumably reoxidized by a component of the endogenous electron transport chain of chloroplasts[18]. Since the switching from aerobic to cyclic photophosphorylation occurs with cofactors with a redoxpotential around and below zero[9] (table 3 see also [16 and 17]), one might conclude, that the endogenous oxidizing component has a redoxpotential of about zero volt. Two endogenous compounds of chloroplasts, as far as discovered, have such a redoxpotential: plastoquinone and cytochrome b_6. It is interesting, that Kamen also found an optimum in bacterial photophosphorylation at a redoxpotential of zero volt[19].

The conclusion, that a true cyclic photophosphorylation is possible in broken chloroplasts, has been questioned because stimulation of photophosphorylation (with suboptimal cofactor concentrations) by oxygen has been observed. Also, isotope experiments showed fast O_2-exchange between air and water in cyclic photophosphorylation with some of the original cofactors[2o]. However, all this shows is that the reduced cofactor of cyclic photophosphorylation is preferentially reacting with oxygen, if present, rather than with the endogenous oxidizing component in the chloroplasts. Whereas oxygen is able to react with very small amounts of a hydroquinone, a certain concentration of the cofactor of true cyclic photophosphorylation has to be used to saturate the reaction with the endogenous oxidizing component of the chloroplasts. Also, a certain specifity in the constitution of a cofactor of cyclic photophosphorylation

should be expected (whereas almost any redox-catalyst will be active in the presence of oxygen). Inhibition studies with low concentrations of DCMU and KCN[12,21,22] and the separation of a cyclic photophosphorylation system from the oxygen evolution system[23], seems to support strongly the view, that true cyclic photophosphorylation exists, at least when vitamin K_3 or PMS as cofactors are used. Of course, this does not mean that cyclic photophosphorylation, as observed in isolated chloroplasts, is also a physiological reaction.

Very recently Arnon showed, that ferredoxin can act, under certain conditions, as a cofactor of cyclic photophosphorylation[24]. The stimulation of this cyclic photophosphorylation by DCMU[24] is reminiscent of the cyclic system with DCPIP as catalyst[22]. As table 4 shows, DCPIP is effective as a cofactor of a photosynthetic ATP formation in the absence of oxygen. This ATP formation is not only insensitive to high concentrations of DCMU, but is actually stimulated by it. Photophosphorylation in the presence of oxygen is, however, inihibited. (Since the DCPIP must first be reduced in a DCMU-sensitive Hill reaction, DCMU was added after 1 min pre-illumination). Cyclic photophosphorylation with DCPIP as catalyst has recently been confirmed in several laboratories[25-27].

additions to o,3 µmol DCPIP	µmoles ATP formed in nitrogen	air
---	1,5	6,3
+ $1o^{-4}$m DCMU	5,3	o,1

Table 4: DCPIP as cofactor of cyclic photophosphorylation (conditions as in table 1, 15 min light. DCMU was added after 1 min pre-illumination).

3. Quinones in H_2O_2 formation

Warburg showed that in aerobic photophosphorylation the hydroquinone, acting as a catalyst, is reoxidized by O_2 under formation of H_2O_2[14]. Since endogenous catalase of the chloroplasts would decompose most of this H_2O_2, a catalase inhibitor has to be added in order to observe H_2O_2 accumulation. KCN, aminotriazole or diethyldithiocarbamate can be used, since these compounds do not interfere with the photosynthetic reactions[22]. $1o^{-3}$m KCN is the most convenient inhibitor. Instead of inhibiting the endogenous catalase, an ethanol/catalase trap for H_2O_2 may be added[10,28]. The reaction sequence of aerobic photophosphorylation in the presence of KCN is then:

$$Q + H_2O + ADP + P_i \longrightarrow QH_2 + 1/2\ O_2 + ATP$$

$$QH_2 + O_2 \longrightarrow Q + H_2O_2$$

sum: $H_2O + 1/2\ O_2 + ADP + P_i \longrightarrow H_2O_2 + ATP$

The ratio of ATP and H_2O_2 formation to O_2 uptake is 1 : 1 : o,5 [14,22].

The Hill reaction with a quinone may therefore be followed not by oxygen evolution, but by oxygen uptake, if the experiment is done in air and $1o^{-3}$m KCN is added. Only catalytic amounts of a quinone are required, which is often important, when substrate amounts of a quinone are inhibitory or insoluble.

H_2O_2 formation by illuminating chloroplasts and its stimulation by quinones is long known as Mehler reaction[29]. It seems, however, somewhat misleading, to speak of oxygen as a Hill reagent, when it is only a variant of a Hill reaction with a quinone.

4. The photooxidation of hydroquinones

It is proper to assume, that the reaction between a hydroquinone and oxygen under formation of H_2O_2 in aerobic photophosphorylation is an autoxydation, particularly if the hydroquinone has a low redoxpotential. This is not correct, however, in the case of the oxidation of hydroquinones with rather positive redoxpotentials, since these are not, at p_H 8, readily autoxidizable. Still, p-hydroquinone and even better, chlorogenic acid or dopamin are, as shown in table 2, excellent cofactors of aerobic photophosphorylation and these hydroquinones are therefore rapidly oxidized by chloroplasts. The suggestion, that a phenoloxidase might be responsible[14], can be ruled out, since the experiment can be done in the presence of KCN[22], which would inhibit the phenoloxydase.

Table 5 shows, that chlorogenic acid, p-hydroquinone and dopamin (with redoxpotentials above +29o mV) are not substantially oxidized by chloroplasts in the dark, whether KCN is absent or not (there is some phenoloxidase activity, as seen in the dopamin experiment). In the light, however, oxygen is taken up and H_2O_2 accumulates, even and particularly in the presence of KCN (which again inhibits endogenous catalase).

	in the dark		in the light	
	μatoms O taken up	μmoles H_2O_2 formed	μatoms O taken up	μmoles H_2O_2 formed
chlorogenic acid	o	o	1,6	1,o
" + $1o^{-3}$m KCN	o	o	6,4	5,8
p-hydroquinone	o	o	o,8	o,8
" + $1o^{-3}$m KCN			5,2	4,8
dopamin	1,3	o	2,1	o,8
" + $1o^{-3}$m KCN	o	o	7,2	7,o

Table 5: Photooxidation of hydroquinones (5 μmol) by broken chloroplasts (conditions as in table 1; 15 min).

These hydroquinones therefore seem to be photooxidized. This photooxidation is not a chlorophyll sensitized photooxidation for two reasons. 1. The photooxidation is inhibited by 10^{-6}m DCMU and 10^{-4}m o-phenanthroline[30] (see also table 11). 2. Treatment of chloroplasts with a detergent and solubilizing the chlorophyll destroys the ability to photooxidize hydroquinones[30].

The inhibition of this photooxidation by DCMU seems to indicate that the oxygen evolution system of photosynthesis somehow participates. It is difficult to give an explanation. It seems conceivable that a hypothetical peroxyde, which gives off oxygen in usual photosynthesis, is oxidizing the hydroquinone, perhaps via a quinolperoxyde. A mechanism like this has been proposed also by Jagendorf as an explanation for the photooxidation of ascorbate by otherwise unsupplemented chloroplasts[31]. This theory implicates that oxygen is required in order to evolve oxygen, even in normal photosynthesis. Such a hypothesis has been advanced in particular by Schenck on the basis of the behavior of chlorophyll in chemical photoreactions[32]. Certainly more experiments are needed, to support such a view.

5. Quinones in the photooxidation of ascorbic acid

As already mentioned, the photooxidation of o-hydroquinones shows similarities to the photooxidation of ascorbic acid. There are several possible ways, in which ascorbic acid may be oxidized by chloroplasts, which cannot be discussed here in detail (see Jagendorf[33] for a review of the pertaining literature). Stimulation of ascorbic acid oxidation by quinones was studied in several laboratories[34-40, 31]. Wessels concluded that the quinone stimulated photooxidation of ascorbic acid is a chemical, chlorphyll sensitized, photooxidation[34]. Others, however, concluded that ascorbic acid photooxidation proceeds via all or part of the electron transport chain of chloroplasts[35,37]. Quinone stimulated ascorbic acid photooxidation was inhibited by o-phenanthroline in Ikeda's experiments[37]. Substituted p-benzoquinones with a redoxpotential in the range from o till + 200 mV cannot be used for the stimulation of ascorbic acid photooxidation by chloroplasts, since they catalyze already a rapid dark oxidation. Also vitamin K_3 at a concentration of 10^{-3}m catalyzes a chemical dark oxidation of ascorbic acid. More interesting is the stimulation of ascorbic acid oxidation by low concentrations of vitamin K_3 (10^{-5}m) and by anthraquinonesulfonic acid, which occurs only by illumination with a chloroplast system. This stimulation is inhibited by DCMU (table 6), which suggests that the electron transport chain of photosynthesis in chloroplasts or part of it is participating. 10^{-3}m KCN was not inhibiting and was added to prevent H_2O_2 decomposition by the endogenous catalase. Coupled ATP formation in a ratio 1 to 1 to H_2O_2 also supports the view that we are not just dealing with a chlorophyll sensitized photooxidation.

Two explanations for this stimulation of ascorbate oxidation can be offered (see also Jagendorf[33]):

1. The quinone is reduced in a Hill reaction and rapidly autoxidized under formation of H_2O_2. The H_2O_2 (perhaps with the help of a peroxidase) is quickly

oxidizing ascorbic acid. The DCMU sensitivity of the Hill reaction would account for the DCMU inhibition of the overall reaction.

However, at the short exposure time in the experiments of table 6, ascorbic acid is stable in the presence of H_2O_2.

additions to 1o µmol ascorbate	oxygen uptake µatoms	H_2O_2-formation (µmol)	ATP-formation (µmol)
---	o,6	o,5	
o,1 µmol anthraquinonesulfonate	13,5	7,o	7,o
o,1 µmol anthraquinonesulfonate + $1o^{-4}$m DCMU	o,1	o,1	o,1
o,o3 µmol vitamin K_3	12,o	6,2	6,o
o,o3 µmol vitamin K_3 + $1o^{-4}$m DCMU	o,8	o,3	o,1

Table 6: Inhibition of quinone stimulated ascorbate photooxidation by DCMU (conditions as in table 1; 15 min light in air, $1o^{-3}$m KCN per vessel).

2. Ascorbic acid is donating electrons into the electron transport chain at a point before the DCMU inhibition site. The electron is transported to the quinone, which is then autoxidized. This is a mechanism, also proposed by Ikeda[37] and very recently by Chiba[39] and which is in agreement with the experiments of Habermann[38] and Marré[38a]. In this view, ascorbate (Aa) would substitute for water as electron donor at the same site.

$$ADP + P_i + H_2Aa + Q \xrightarrow{light} QH_2 + Aa + ATP$$

$$QH_2 + O_2 \longrightarrow Q + H_2O_2$$

sum: $$ADP + P_i + H_2Aa + O_2 \longrightarrow Aa + H_2O_2 + ATP$$

The ratio of H_2O_2 and ATP formation to O_2 uptake in this reaction type is 1 : 1 : 1 as against the ratio of 1 : 1 : o,5 in aerobic photophosphorylation.

This quinone stimulated ascorbic acid photooxidation differs in many respects from the DCPIP stimulated ascorbate oxidation. The latter is not inhibited by DCMU[4o]. One major change introduced by the addition of DCPIP is the point of entry of the electrons donated. As the work of Vernon[41] and Witt[42] clearly indicated, ascorbate donates electrons via DCPIP into the cytochrome chain.

Explanation 2 would also account for the ascorbate stimulation of cyclic photophosphorylation with certain cofactors. Vennesland[12] showed, that for FMN catalyzed cyclic photophosphorylation catalytic amounts of ascorbate are required and that the ascorbate stimulation is abolished by o-phenanthroline.

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Table 7 indicates, that photophosphorylation catalyzed by indigo-sulfonic acids is like the FMN system stimulated by ascorbate. This stimulation is again DCMU sensitive.

	µmoles ATP formed
o,2 µmol indigo-sulfonic acid	1,1
" " + 2,5 µmol ascorbate	4,4
" " " " + $1o^{-4}$ DCMU	o,8
o,2 µmol indigo-disulfonic acid	1,2
" " + 2,5 µmol ascorbate	6,1
" " " " + $1o^{-4}$ DCMU	1,o
o,1 µmol FMN	1,5
" " + 2,5 µmol ascorbate	7,6
" " " " + $1o^{-4}$ DCMU	1.7

Table 7: Stimulation of cyclic photophosphorylation by ascorbate (conditions as in table 1; 15 min light in N_2).

This might best be explained as a donation of electrons by ascorbate into the electron transport chain before the DCMU block. In the absence of ascorbate, the oxidation of the reduced cofactor by an endogenous oxidizing compound of the chloroplasts is limiting. Ascorbate alone is not a good cofactor, since then the reduction of dehydro- (or monodehydro-) ascorbate is limiting[31]. Against the explanation that ascorbate substitutes for water is, that a stoichiometry of oxygen evolution and TPNH formation is observed also in the presence of ascorbate.

o-Quinones (or rather o-hydroquinones) behave quite different from quinones with negative redoxpotentials in the ascorbate oxidation by chloroplasts. o-Hydroquinones do not stimulate ascorbic acid photooxidation by chloroplasts. On the contrary, ascorbate inhibits the photooxidation of these hydroquinones (table 8).

	O_2 uptake µatoms	H_2O_2 formed µmoles
o,1 µmol chlorogenic acid	3,7	3,1
o,1 µmol chlorogenic acid + 1o µmol ascorbate	o,8	o,6
o,1 µmol catechol	6,5	5,5
o,1 µmol catechol + 1o µmol ascorbate	o,8	o,4
o,1 µmol anthraquinonesulfonate	5,6	6,o
o,1 µmol anthraquinonesulfonate + 1o µmol ascorbate	14,1	6,7

Table 8: Inhibition of o-hydroquinone photooxidation by ascorbate (in comparison with anthraquinone) (15 min light in air; $1o^{-3}$m KCN per vessel).

This might best be explained by a competition of o-hydroquinone and ascorbate for the same site of oxidation. Since no autoxidizable hydroquinone is formed (as it is from anthraquinone) the system is blocked.

6. The action of salicylaldoxime on photosynthetic reactions

Salicylaldoxime is a copper chelating agent, which inhibits copper containing enzymes, like phenoloxydase[43] and cytochromeoxydase[44]. It also inhibits photosynthesis in intact Chlorella[45]. Table 9 shows the influence of salicylaldoxime on various photosynthetic activities in broken chloroplasts. All reactions involving oxygen evolution (ferricyanide and TPN reduction) as well as cyclic photophosphorylation (vitamin K_3 as cofactor) are inhibited by 10^{-2}m salicylaldoxime. The only photosynthetic reaction possible in the presence of salicylaldoxime is the reduction of TPN at the expense of DCPIP/ascorbate.

For comparison the behavior of DCMU, as worked out by Vernon and others[41,42,46] is included in table 9. The difference between DCMU and salicylaldoxime is, that DCMU does not inhibit cyclic photophosphorylation, whereas salicylaldoxime does. In the DCMU experiments, the addition of DCPIP/ascorbate restores TPNH and ATP formation, whereas in the salicylaldoxime experiments coupled ATP formation does not reappear, when TPN is reduced by DCPIP/ascorbate.

According to Vernon[41] and Witt[42] reduced DCPIP reacts with cytochrome f. Since salicylaldoxime does not influence photosynthetic TPN reduction at the expense of DCPIP/ascorbate, the site of inhibition of salicylaldoxime must be before cytochrome f. But since salicylaldoxime inhibits cyclic photophosphorylation, its site of inhibition would be after plastoquinone and the second light reaction (see scheme). The phosphorylation site (either in cyclic or non-cyclic photophosphorylation) cannot be between cytochrome f and TPN, since the reaction sequence: ascorbate → DCPIP → cytochrome f → light → TPN is not coupled in the presence of salicylaldoxime. Witt already argued for reasons of redoxpotential that the phosphorylation site has to be between plastoquinone and cytochrome f[47]. In the DCMU experiments, where the reduction of TPN by DCPIP/ascorbate is coupled to ATP formation[46], one has to assume, that DCPIP does not react with cytochrome f but with a compound, located in the electron transport chain before the phosphorylation site, possibly plastoquinone or even with a compound (Y) before the second light reaction, as suggested by Witt[42].

The inhibition of photosynthetic reactions in chloroplasts by salicylaldoxime seems interesting: for if it is accepted that salicylaldoxime as copper chelating agent inhibits a copper enzyme in these experiments with chloroplasts, then the site of salicylaldoxime inhibition would indicate the location of this copper enzyme in the electron transport chain of photosynthesis.

Such a copper enzyme has already been isolated from chloroplasts. Katoh named it plastocyanine and drew attention to its possible significance in

			µatoms O evolved	µmoles TPNH	µmoles ATP
2o µmol ferricyanide			7,o		6,9
"	"	+ $1o^{-5}$m DCMU	o,1		o,1
"	"	+ $1o^{-2}$m salicylaldoxime	o,4		o,1
"	"	+ $1o^{-2}$m KCN	o,5		o,5
o,3 µmol vitamin K_3					8,2
"	"	+ $1o^{-4}$m DCMU			7,4
"	"	+ $1o^{-2}$m salicylaldoxime			o,8
"	"	+ $1o^{-2}$m KCN			o,8
6 µmol TPN		+ 1o µmol ascorbate	2,8	2,9	2,4
"	"	+ $1o^{-5}$m DCMU + 1o µmol ascorbate	o,2	o,1	o,1
"	"	+ $1o^{-5}$m DCMU + 1o µmol ascorbate + o,2 µmol DCPIP	o,4	2,o	2,5
"	"	+ $1o^{-2}$m salicylaldoxime	o,4	o,3	o,1
"	"	+$1o^{-2}$m salicylaldoxime + 1o µmol ascorbate	o,6	1,2	o,1
"	"	+$1o^{-2}$m salicylaldoxime + 1o µmol ascorbate + o,2 µmol DCPIP	o,3	2,3	o,1

Table 9: Inhibition of photosynthetic reactions in broken chloroplasts by salicylaldoxime and KCN in comparison to DCMU (conditions as in table 1, 15 min light in nitrogen).

photosynthesis[48]. Plastocyanine is inhibited by salicylaldoxime[48]. Its properties are not against the site of function we are suggesting. Therefore plastocyanine might be identical with the copper enzyme, which is inhibited by salcylaldoxime in isolated chloroplasts. The site of inhibition of salicylaldoxime might also be at the compound E, Witt is proposing at that particular point[47]. Further experiments are needed to establish the role of copper and its function at the suggested site in photosynthesis.

Table 9 shows that rather high concentrations of KCN behave just like salicylaldoxime. We think therefore, that the inhibition of photosynthesis by $1o^{-2}$m KCN, as observed by Warburg[49], Jagendorf[31], Schwartz[5o] and Vennesland[51] might be due to the inhibition of the copper enzyme.

The influence of salicylaldoxime on photosynthetic reactions of chloroplasts can also be shown in broken chloroplasts, which were treated shortly with $5 \cdot 1o^{-2}$m salicylaldoxime and then washed once to remove most of the salicylaldoxime during the actual photosynthesis experiment. In such "salicylaldoxime treated" chloroplasts again only the photosynthetic reduction of TPN with DCPIP/ascorbate as electron donor is possible; cyclic photophosphorylation and O_2 evolution are blocked[52]. Broken chloroplasts treated shortly with $3 \cdot 1o^{-2}$m KCN behave as salicylaldoxime treated chloroplasts[52].

These salicylaldoxime treated chloroplasts show a number of interesting properties besides those, already mentioned.

7. Photooxidations in salicylaldoxime treated chloroplasts

It is seen already in table 9 that in the presence of salicylaldoxime ascorbate alone without the addition of DCPIP is able to restore TPNH formation to a certain extent (in DCMU experiments this is not possible). The same is also true in salicylaldoxime treated chloroplasts[52]. Ascorbate seems to enter the electron transport chain in these experiments not before the DCMU inhibition site (as in the photooxidation experiments in intact chloroplasts, as discussed in chapter 5), but even after the salicylaldoxime inhibition site, possibly at cytochrome f. Loosening of the copper enzyme, usually tightly coupled to cytochrome f by salicylaldoxime seems to allow access of the ascorbate to the cytochrome f without the mediation of DCPIP.

This change of the point of entry of ascorbate into the chain of electron carriers by treating chloroplasts with salicylaldoxime is supported by the different behavior of quinone stimulated ascorbate photooxidation. In untreated broken chloroplasts (P_{1s3} in table 1o) the photooxidation of ascorbate is inhibited by DCMU (as discussed in chapter 5), in salicylaldoxime treated chloroplasts it is not (table 1o).

The behavior of o-hydroquinone photooxidation also changes. In broken chloroplasts the photooxidation of o-hydroquinones is inhibited by DCMU[3o], in salicylaldoxime treated chloroplasts it is not (table 11). This distinguishes treatment of chloroplasts with salicylaldoxime (or KCN) from that with detergents.

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In detergent treated chloroplasts ascorbic acid photooxidation is enhanced[36] and not anymore inhibited by o-phenanthroline[39], but the photooxidation of o-hydroquinone is completely inactivated[30].

additions (o,1 µmol) to 1o µmol ascorbate	P_{1s3}		salicylaldoxime treated P_{1s1}	
	O_2 uptake µatoms	H_2O_2 formed µmoles	O_2 uptake µatoms	H_2O_2 formed µmoles
+ vitamin K_3	11,4	5,7	5,2	2,3
+ vitamin K_3 + $1o^{-4}$m DCMU	o,7	o,1	4,6	2,3
+ anthraquinonesulfonate	12,9	5,7	5,7	3,1
+ anthraquinonesulfonate + $1o^{-4}$m DCMU	o,4		5,6	2,1

Table 1o: Ascorbate photooxidation in normal and salicylaldoxime treated chloroplasts (P_{1s1}, containing 4 mg chlorophyll, were suspended in 3 ml cold $5 \cdot 1o^{-2}$m salicylaldoxime, spun down after 2 min, washed once with water and resuspended in 2 ml water. Further treatment as in table 1, 15 min light in air, $1o^{-3}$m KCN per vessel).

	µatoms oxygen taken up in	
	P_{1s3}	salicylaldoxime treated P_{1s1}
5 µmol chlorogenic acid	3,3	1,7
" " + $1o^{-4}$m DCMU	o	1,7
5 µmol DOPA	6,5	6,o
" " + $1o^{-4}$m DCMU	o,7	7,1
1o µmol ascorbate + o,1 µmol anthraquinone sulfonate	8,3	4,3
" " " + $1o^{-4}$ DCMU	o,6	4,2

Table 11: Photooxidation of o-hydroquinones in normal and salicylaldoxime treated chloroplasts (compared with ascorbate oxidation) (conditions as in table 1o).

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8. p-Hydroxylation of salicylaldoxime by chloroplasts

The salicylaldoxime experiments, described so far, were done at a concentration of 10^{-2}m. In air at a concentration of less than 10^{-3}m, salicylaldoxime behaves quite different. It is then not an inhibitor, but a cofactor of photophosphorylation in chloroplasts[53]. Of a number of substituted phenols tested, only salicylaldehyde and its oxime were active as cofactors of an aerobic photophosphorylation (table 12).

	µmoles ATP formed
---	1,0
10^{-2} m salicylaldoxime in nitrogen	0,5
10^{-3} m " in "	0,5
10^{-2} m " in air	0,5
10^{-3} m " "	5,5
10^{-4} m " "	6,3
10^{-3} m salicylaldehyde "	3,9
" o-cresol "	1,1
" m-cresol "	1,1
" p-cresol "	0,9
" salicylalcohol "	1,4
" salicylic acid "	1,1
" gentisinalcohol "	6,5
" gentisinaldehyde "	6,9
" gentisinic acid "	5,5

Table 12: Aerobic photophosphorylation with substituted phenols (conditions as in table 1).

Since salicylaldehyde (or its oxime) is not a reversible redoxcatalyst, one has to assume, that it can be converted into one by the chloroplast system. It is, indeed, possible to isolate gentisinaldehyde as its dinitrophenylhydrazone, after salicylaldehyde has been incubated in air with chloroplasts in the light[53]. This indicates that salicylaldehyde and its oxime are hydroxylated in the p-position to a compound, which can now be reversibly oxidized and reduced and which is the actual cofactor of the photophosphorylation. As seen in table 12, gentisinaldehyde is quite an active cofactor of photophosphorylation in air. A somewhat more detailed examination of the hydroxylation system suggested, that a peroxydase reaction is responsible for the hydroxylation[53].

The inhibitor salicylaldoxime is by this hydroxylation converted into a cofactor of photophosphorylation. This is reminiscent of Wessel's experiments with dinitrophenol. This inhibitor of photophosphorylation is, by reduction to an aminophenol by illumination with chloroplasts, also converted into a cofactor[54].

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9. Benzoquinones in the reactivation of petrolether extracted chloroplasts

As already mentioned, plastoquinone = dimethyl-solanosyl-p-benzoquinone is an endogenous quinone of chloroplasts[4]. Its importance for the photosynthetic activity of chloroplasts became apparent, when Bishop showed that the ferricyanide (and DCPIP) reduction in petrolether extracted chloroplasts was plastoquinone dependent[5]. A number of groups have now investigated the system of Bishop i.e. the properties of petrolether extracted chloroplasts in more detail[55-60]. The results are, that all known photosynthetic reactions of chloroplasts—the Hill reaction with different acceptors (ferricyanide[5,55,60], DCPIP[5], benzoquinones[56] and TPN[56,59])—are inactivated and become plastoquinone dependent, when the chloroplasts were exhaustively extracted with petrolether or acetone. The only exception is the reduction of TPN by DCPIP/ascorbate, which seems to be independent of plastoquinone[56,59] (see however Crane[60]). This provides some indication as to the location of endogenous plastoquinone in the electron transport chain of chloroplasts. In agreement with Witt's interpretation of experiments, which gave more direct spectroscopic evidence[57], plastoquinone is probably the acceptor of the second light reaction and is situated before the cytochrome chain.

We have pointed out, however, that incomplete extraction of plastoquinone leads to a somewhat different picture[56]. By extraction of 70% of the endogenous plastoquinone (which can easily be accomplished by an only short treatment of chloroplasts with petrolether), only the reduction of ferricyanide and of o-quinones is impaired, but not the reduction of TPN and p-benzoquinones[56]. We have concluded from this, that there is a second site of plastoquinone in the electron transport chain[56]. This second site has to be in a sidepath to the main chain (leading to TPN) after the first light reaction (see scheme) and is participating only in ferricyanide reduction. This second plastoquinone site has recently been confirmed by Witt by direct spectroscopic observations at 260 mu[58].

Crane recently suggested also several plastoquinone sites in photosynthesis[60]. The higher sensitivity of the Hill reaction towards UV-light as compared to cyclic photophosphorylation, noticed by Avron[61], and the stimulation of photosynthetic reactions of chloroplasts by the further addition of plastoquinone[61,62] (surprising in view of the high plastoquinone content of chloroplasts) might also be explained by two plastoquinone sites, only one of which is of physiological importance.

The two plastoquinone sites are different also in the specifity by which they can be reactivated after petrolether extraction of the endogenous plastoquinone[56]. Table 13 shows that after incomplete extraction of plastoquinone (removal of plastoquinone at site 2), the reduction of ferricyanide, vitamin K_3 and anthraquinone-sulfonic acid is impaired and shows a stimulation by the addition of plastoquinone-45, whereas this is not the case with the reduction of TPN and the two substituted benzoquinones.

	µmol electrons transferred	
	without	with the addition of o,2 µmol plastoquinone
5 µmol TPN	2,o	2,1
1o µmol ferricyanide	o,1	2,2
o,2 µmol 2,3-dimethyl-benzoquinonebutyrate	1,8	2,2
o,2 µmol 2,3-dimethoxy-methylbenzoquinone	2,1	2,2
o,2 µmol vitamin K_3	o,1	2,8
o,2 µmol anthraquinone-sulfonate	o,1	2,5

Table 13: Plastoquinone dependence of the reduction of TPN, ferricyanide and quinones in petrolether extracted chloroplasts (only 7o% of endogenous plastoquinone removed). o,3 ml dialyzed, watersoluble chloroplast extract was added in the TPN experiment; quinone reduction was measured by H_2O_2 formation in the presence of $1o^{-3}$m KCN. 15 min light in air (56).

The ferricyanide system in such incomplete extracted chloroplasts can be reactivated by numerous p-benzoquinones, some of which are shown in table 14. There seems to be no structural requirements in the reactivation of the second plastoquinone site, except that it has to be a substituted p-benzoquinone.

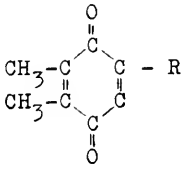
addition of o,2 µmol	µmol ferricyanide reduced
---	o,8
p-benzoquinone	1,o
2,3-dimethoxy-p-benzoquinone	3,8
2,3-dimethoxy-methylbenzoquinone	3,7
2,3-dimethyl-p-benzoquinone	3,8
2,3-dimethyl-benzoquinone-butyric acid	4,1
trimethyl-benzoquinone	4,2
p-benzoquinone-2,5-diacetic acid	4,4
vitamin K_3	o,9

Table 14: Reactivation of ferricyanide reduction in petrolether extracted chloroplasts (only 7o% of endogenous plastoquinone removed). 15 min light in N_2.

Table 15 shows that after exhaustive extraction of plastoquinone the TPN system is now also plastoquinone dependent (at site one). The reactivation of this plastoquinone site is very specific. Only dimethyl-substituted benzoquinones with an isoprenoid side chain of at least 5 C-atoms are active. Allyl- as well as butyrate substitution in position 5 show no activity.

addition of o,2 µmol of	µmol TPNH formed
---	o,5
plastoquinone-45	3,o
plastoquinone-15	2,7
plastoquinone-5	2,9
2,3-dimethyl-phytylbenzoquinone	3,2
2,3-dimethyl-5-allylbenzoquinone	o,6
2,3-dimethyl-benzoquinone	o,8
2,3-dimethoxy-methylbenzoquinone	o,7
2,3-dimethyl-benzoquinone-butyric acid	o,5
ubiquinone-5o	o,5

Table 15: Reactivation of TPN reduction by quinones in petrolether extracted chloroplasts (exhaustively extracted, o,3 ml dialyzed, watersoluble chloroplast extract added, 15 min light in N_2).



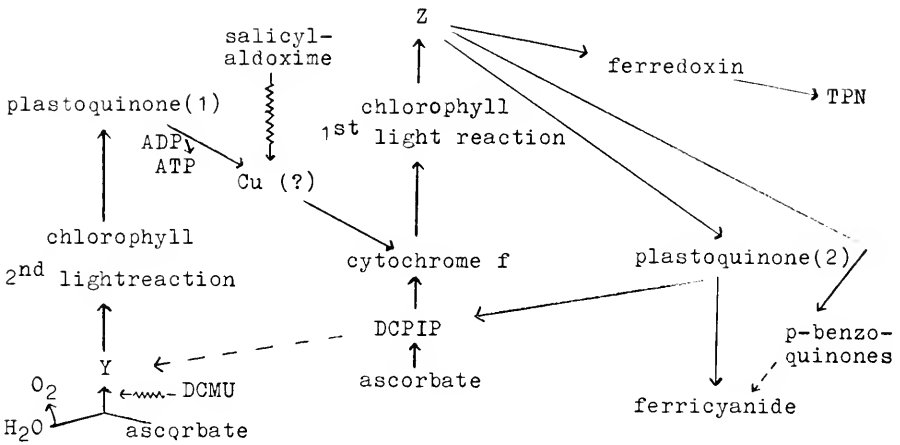
R = $-CH_2-CH=C(CH_3)_2$ (-5-dimethyl-allyl (plastoquinone-5)); $-CH_2-CH=CH_2$ (-5-allyl); $-CH_2-CH_2-CH_2-COOH$ (-5-butyric acid)

plastoquinone-45 activity: yes; no; no

Besides two methyl-groups in position 2 and 3, a substitution at position 5 by $-CH_2-CH=C-(CH_3)_2$ seems to be essential for the activity at the first plastoquinone site in the electron transport chain of photosynthesis.

Krogmann[55] and Crane[6o] also showed that different photosynthetic reactions of chloroplasts have different structural requirements in their reactivation, after endogenous plastoquinone has been extracted.

The following scheme is to indicate the proposed two sites of plastoquinone function and the site of salicylaldoxime inhibition as discussed in the preceding chapters.



Scheme of photosynthetic electron transport in broken chloroplasts

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PHOTOSYNTHETIC ELECTRON TRANSPORT AND PHOSPHORYLATION IN CHLOROPLASTS

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The type of photosynthetic phosphorylation first found in chloroplasts--the type now called cyclic photophosphorylation--in which the sole product of the reaction is ATP, and in which no hydrogen (electron) donor or acceptor is consumed, yielded no experimental evidence for a light-induced electron transport coupled with phosphorylation (1,2). Direct experimental evidence, as distinguished from supposition, for a coupling between photosynthetic phosphorylation and photosynthetic (light-driven) electron transport in chloroplasts came in 1957 with the finding of what we now call noncyclic photophosphorylation (3). Here the formation of ATP was linked with a thermodynamically "uphill" hydrogen (electron) transfer from water to TPN (or ferricyanide)--a transfer that was accompanied by a stoichiometric oxygen evolution.

The early hypotheses linking photophosphorylation with photosynthetic electron transport centered on the photolysis of water as a common primary photochemical event in cyclic and noncyclic photophosphorylation (4). But since 1959 our work has been guided by an "electron flow" hypothesis which limits the photooxidation of water and the resulting evolution of oxygen to noncyclic photophosphorylation (5). The common primary photochemical event (coupled with ATP formation) in both cyclic and noncyclic photophosphorylation is now envisaged as an electron transfer from excited chlorophyll to a primary electron acceptor molecule and thence, with the aid of appropriate enzyme systems, either to TPN (noncyclic) or back to chlorophyll via the cytochrome chain (cyclic electron flow) (5,6).

In 1961 this hypothesis was further elaborated (7) to accommodate the experimental separation of noncyclic photophosphorylation in chloroplasts into two partial reactions: (a) ATP formation without oxygen evolution but coupled with the photoreduction of TPN by the ascorbate-DPIP couple, and (b) photooxidation of water to molecular oxygen (7). With the separation of these two reactions there was also preliminary evidence that photoproduction of oxygen is catalyzed by a pigment system different from

that required for the photoreduction of TPN (8).

The aim of this article is to summarize the work in our laboratory since 1961 which extends and supports the electron flow concept of photophosphorylation in chloroplasts. The discussion will include experiments which led to: (a) The identification of ferredoxin as the most electronegative electron carrier isolated so far from chloroplasts and the elucidation of its role in photosynthetic electron transport. This followed a series of experiments on photoreduction of methyl viologen and photoproduction of hydrogen gas by chloroplasts. (b) Correlations between pigment function and photochemical activity of spinach chloroplasts and algal chromatophores, and (c) identification of the position of plastoquinone in the noncyclic electron transport chain of chloroplasts.

Some of the material given here was published in more detail elsewhere (9-13); other reports are in preparation. Extensive reviews of earlier work from this and other laboratories are available (6,14).

Position of plastoquinone in the noncyclic electron transport chain. Crane (15) found that plastoquinone is localized in chloroplasts and Bishop (16) and Krogmann (17) have shown that it is required for the photoreduction of ferricyanide or 2,6-dichlorophenol indophenol by isolated chloroplasts. Fig. 1 shows that, after extraction of plastoquinone, chloroplasts lost the ability to photoreduce TPN when water (OH^-) was the electron donor but not when the ascorbate-DPIP couple replaced water as the electron donor system (18).

Plastoquinone thus appears to be required in that portion of the photosynthetic electron transport chain in chloroplasts which is concerned with the photooxidation of water to molecular oxygen and which has been identified by Losada et al. (7) as the first of the two light reactions that jointly bring about the transfer of electrons from water to TPN. Plastoquinone may thus be the endogenous chloroplast factor which occupies the position marked as "A" in the noncyclic electron flow scheme of 1961 (Fig. 3 in ref. 7). Similar conclusions about the position of plastoquinone in the chloroplast electron transport chain were reached by Witt et al. (19).

Photoreduction of methyl viologen. According to the electron flow hypothesis, the primary electron carrier, common to the cyclic and noncyclic electron flow, must be able to reduce not

only TPN (E'_o = -320 mV, pH 7) but also strongly electronegative redox dyes such as methyl viologen (E'_o = -455 mV, pH 7), a dye which was shown by Jagendorf and Avron (20) and Hill and Walker (21) to catalyze cyclic photophosphorylation.

It would follow from these considerations that an accumulation of photoreduced methyl viologen, although never before demonstrated, should be possible if its reoxidation by oxygen or by the cyclic electron flow mechanism of chloroplasts is prevented. This was experimentally shown by Mitsui et al. (22). Table 1 shows that illuminated chloroplasts reduced methyl viologen in the presence of cysteine and dichlorophenolindophenol (DPIP). No reduction of methyl viologen was observed in the dark or after boiling the chloroplasts for 5 min.

Table 1

Photoreduction of Methyl Viologen by Chloroplasts (22)

	Methyl viologen reduced (μmoles/hr/mg chl)
Complete system	54.2
DPIP omitted	5.8
Cysteine omitted	0.2

Water was not the electron donor since the system was incapable of evolving oxygen, with or without added CMU. The rate of photoreduction of methyl viologen was greatly decreased when DPIP was omitted from the reaction mixture. Thus, DPIP rather than cysteine appeared to be the effective electron donor. However, the function of cysteine was not limited to donating electrons *via* DPIP since ascorbate, which can also reduce DPIP, did not replace cysteine in the same system. Only after a mild heat treatment (50° C for 10 min.), which greatly reduced the total photoactivity of chloroplasts, were chloroplasts able to use the ascorbate-DPIP couple as an electron donor for the photoreduction of methyl viologen (at a low rate).

The effectiveness of cysteine is explained by its inhibition of the cyclic electron flow by which reduced methyl viologen can be reoxidized by chloroplasts. As shown in Table 2, cysteine strongly inhibited cyclic photophosphorylation catalyzed by methyl viologen, menadione or FMN but not the "shortcut" cyclic photophosphorylation pathway catalyzed by phenazine methosulfate (6). The last observation suggests that the inhibitory effect of cysteine on cyclic photophosphorylation was not related to an activation of ATPase (23) since in that case all photophosphory-

lations would be expected to be affected.

Table 2
Inhibition of Cyclic Photophosphorylation with Cysteine (22)

Additions	μmoles ATP formed
Methyl viologen	5.2
Methyl viologen, cysteine	0.3
Vit. K_3	6.6
Vit. K_3, cysteine	0.2
FMN	4.8
FMN, cysteine	0.4
PMS	3.6
PMS, cysteine	3.6

Photoproduction of hydrogen gas by chloroplasts. The ability of spinach chloroplasts to photoreduce methyl viologen--a dye used as an electron carrier for hydrogenase (24)--suggested that spinach chloroplasts would also be capable of photoproducing hydrogen gas, if they were supplied with a hydrogenase (which they lack) and if oxygen production, usually deleterious to hydrogenase activity, were suppressed.

Photoproduction of hydrogen gas by spinach chloroplasts supplemented with bacterial hydrogenases was demonstrated by Mitsui and Paneque (25-27). When photoproduction of oxygen gas was suppressed and cysteine-DPIP (Fig. 2) was used instead of water as the electron donor system, spinach chloroplasts evolved hydrogen gas in the light. The photoproduction of hydrogen gas was accompanied by formation of ATP (Fig. 3).

Ferredoxin and the equivalence of light and H_2 for TPN reduction. At first, photoproduction of H_2 by chloroplasts was carried out with the aid of a hydrogenase isolated from Chromatium (25,26) or Desulfovibrio desulfuricans (26). In both these cases photoproduction of H_2 required the addition of methyl or benzyl viologen (24). However, with a crude hydrogenase from Clostridium pasteurianum no addition of viologen dye was required for the photoproduction of hydrogen gas by chloroplasts (27). The cell-free extract of C. pasteurianum contained an electron carrier which brought about a photoproduction of hydrogen gas by chloroplasts. This was consistent with the isolation by Mortenson et al. (28) of a natural electron-transferring factor in C. pasteurianum, named by them ferredoxin, which, in that organism, couples

pyruvic dehydrogenase with hydrogenase in the production of H_2 from pyruvate.

Aside from photoproduction of H_2, Tagawa and Arnon (9) found that clostridial ferredoxin catalyzed the photoreduction of TPN by spinach chloroplasts without the participation of the "photosynthetic pyridine nucleotide reductase" (PPNR) of San Pietro and Lang (29), which has become widely accepted as the specific chloroplast enzyme required for the photoreduction of TPN. Moreover, in the presence of added bacterial ferredoxin and hydrogenase, isolated chloroplasts reduced TPN in the dark with hydrogen gas but without PPNR (see Fig. 5 in ref. 9). Thus, the enzymic apparatus of chloroplasts was found to be able to reduce TPN independently of PPNR and light.

These results indicated that the true pyridine nucleotide reductase enzyme of chloroplasts was not a component of the PPNR preparation but a component of the remaining chloroplast fraction. A re-examination of the TPN-reducing system of chloroplasts, described in more detail elsewhere (9,30), proved this interpretation to be correct. The TPN reductase proper, which had also diaphorase (31) and transhydrogenase (32,33) activities, was found to be localized in the flavoprotein fraction of chloroplasts (9). The flavoprotein reductase, which was also isolated by Gewitz and Voelker (34) and Davenport (35), was recently crystallized (Fig. 4) by Shin et al. (36,30). The enzyme reduces TPN either in the light or in the dark with H_2 (plus hydrogenase) and requires in either case the collaboration of an electron carrier: bacterial or chloroplast ferredoxin.

Chloroplast ferredoxin was isolated and crystallized by methods similar to those used for bacterial ferredoxin (9). Chloroplast ferredoxin proved to be a non-heme iron protein, localized in chloroplasts, and was similar to Clostridium ferredoxin in having a redox potential (E'_o = -432 mV, pH 7.55) close to that of the hydrogen electrode and in undergoing reversible oxidation-reduction that was measured by spectral changes (9). As shown in Table 3, the protein from spinach chloroplasts had an iron content of 0.89 per cent, which, on the basis of a molecular weight of 13,000, indicates two atoms of iron per mole.

Spinach ferredoxin proved to be the same substance as PPNR, the methaemoglobin reducing factor of Davenport, Hill and Whatley (37), the TPN-reducing factor of Arnon, Whatley and Allen (38) and the red enzyme of Gewitz and Voelker (34) [see review (30)]. The presence of iron in PPNR or the red enzyme was recently re-

ported by Horio and Yamashita (39), Katoh and Takamiya (40), Fry and San Pietro (41) and Gewitz and Voelker (34). Fry and San Pietro (41) found that the iron in this protein is associated with "labile" sulfide groups. The presence of "labile" sulfide groups in the protein was also independently found by Gewitz and Voelker (34).

Table 3
Iron Analysis of Spinach Ferredoxin
(Tagawa, Chain and Arnon, 1963)

Ferredoxin Used (mg)	Fe found (μg)	Per cent Fe	Minimum M.W.
1.0	8.6	0.86	6,490
2.0	17.9	0.90	6,200
3.7	33.3	0.89	6,260
7.4	64.4	0.87	6,420

The chemical similarities and the functional interchangeability of bacterial and chloroplast ferredoxin in the photoreduction of TPN (9) suggest that these two substances, although not identical, belong to a family of ferredoxins. Ferredoxins appear to function as electron carriers that transfer to appropriate enzyme systems the most "reducing" electrons in cellular metabolism, that is, electrons at a potential of about -420 mV. These come from two sources: hydrogen gas (or substrates producing H_2) or illuminated chloroplasts.

The role of ferredoxins in photosynthetic electron transport and in utilization and production of hydrogen gas is diagrammatically represented in Fig. 5. In this scheme, crystalline spinach ferredoxin (Fig. 6) was found to be replaceable by one of several crystalline ferredoxins: that from Clostridium pasteurianum (9) and those from the photosynthetic bacterium Chromatium (Fig. 7) and the blue-green alga, Nostoc muscorum (Fig. 8).

Separation of the light and dark reactions in photoreduction of TPN. The recognition of the role of ferredoxin in the TPN-reducing system was followed by the physical separation of the photoreduction of TPN by illuminated chloroplasts into two steps: (1) a photochemical reduction of ferredoxin by chloroplasts in the absence of TPN, followed by (2) the dark reoxidation of reduced ferredoxin by chloroplasts to which TPN was added (42).

The results, summarized in Table 4, show that two moles of

reduced ferredoxin are required to reduce one mole of TPN. Thus, the oxidation-reduction of a ferredoxin molecule involves a transfer of a single electron. In the absence of evidence to the contrary, it is attractive to assume that the photoreduction of ferredoxin is the terminal photochemical act of chloroplasts following photon capture--an act that involves a transfer of an electron from excited chlorophyll to the electron acceptor molecule in chloroplasts.

Table 4

Stoichiometry of Photoreduction of Spinach Ferredoxin and its Subsequent Reoxidation by TPN in the Dark (10)

	μmoles
Ferredoxin (Fd) photoreduced	0.102
Fd reoxidized by TPN in the dark	0.106
TPN reduced	0.047

Ferredoxin and photophosphorylation. If the photoreduction of ferredoxin by chloroplasts is the terminal photochemical act of chloroplasts, then it follows that photoreduction of ferredoxin should be a common feature of the electron flow pathways of either cyclic or noncyclic photophosphorylation. The two pathways would differ in the electron acceptors beyond ferredoxin. In the case of noncyclic photophosphorylation, the electrons from ferredoxin would be transferred by the flavoprotein reductase to TPN, whereas in the case of cyclic photophosphorylation they would "cycle" back to "electron-deficient" chlorophyll molecules via a chain of endogenous electron carriers (6).

A requirement for ferredoxin [then called "TPN-reducing factor (38)] for noncyclic photophosphorylation coupled with TPN reduction was indeed already observed when this process was first discovered (3) and this requirement has since been further documented (43-47). Evidence was also obtained several years ago (38) for a requirement of a "TPN-reducing factor" (i.e., ferredoxin) in what is now called pseudocyclic photophosphorylation (8) by chloroplasts. Moreover, Forti and Jagendorf (48) and Black et al. (49) found that under aerobic conditions ferredoxin ("PPNR") stimulates an endogenous photophosphorylation which proceeds in the absence of added cofactors, but is dependent on and consumes, molecular oxygen as the terminal electron acceptor.

There was no evidence, however, that ferredoxin catalyzes an anaerobic, cyclic photophosphorylation, when oxygen evolution is effectively inhibited by the presence of CMU. Such evidence was

recently obtained by Tagawa et al. (11) who found that ferredoxin catalyzes an anaerobic cyclic photophosphorylation in chloroplasts which proceeds in the presence of CMU and without the addition of other cofactors. A notable feature of this endogenous, ferredoxin-catalyzed cyclic photophosphorylation, which distinguishes it from other types of cyclic photophosphorylation in chloroplasts, is its sensitivity to antimycin A and to low concentrations of dinitrophenol (11,12). Since, in mitochondria antimycin A inhibition is considered to be indicative of the participation of cytochrome b in electron transport (50,51), the sensitivity to antimycin A suggests a possible participation of the cytochrome b_6 component of chloroplasts (52,53) in the ferredoxin-catalyzed cyclic photophosphorylation.

A previously puzzling feature of cyclic photophosphorylation in isolated chloroplasts, a feature which distinguished it from cyclic photophosphorylation in bacterial chromatophores, was a dependence on an added electron carrier such as vitamin K or phenazine methosulfate. A possible, though heretofore experimentally unsupported, explanation of this difference was that chloroplasts, but not chromatophores, lost a soluble constituent in the process of isolation. The recent findings point to chloroplast ferredoxin as being the water-soluble constituent of cyclic photophosphorylation which is, at least in part, lost from chloroplasts when they are removed from the cell. However, it is still premature to say what role bacterial ferredoxins play in the mechanism of bacterial photophosphorylation.

As previously mentioned, ferredoxin appears to be a junction in chloroplasts for the electron transport systems that lead to either cyclic or noncyclic photophosphorylation. Since we now know that TPN is required for noncyclic photophosphorylation but not for cyclic photophosphorylation, it follows that when the photoreduced ferredoxin is reoxidized by TPN (via the flavoprotein reductase), noncyclic photophosphorylation would result. When oxidized TPN is unavailable as an electron acceptor, the photoreduced ferredoxin would be reoxidized (directly or indirectly) by a cytochrome component of the grana, and cyclic photophosphorylation would result. It is thus possible to envisage that the availability of TPN as an electron acceptor might serve as a physiological regulator between cyclic and noncyclic photophosphorylation. Evidence for this view has recently been reported (11).

Analysis of chloroplast reactions with monochromatic light. The photoreduction of ferredoxin and the resultant cyclic or

noncyclic photophosphorylation were found to be basically independent of photoproduction of oxygen by chloroplasts (12). To demonstrate this independence it was necessary to use special experimental devices such as inhibitors of oxygen evolution, anaerobic conditions and, most recently, monochromatic light (12) at a wavelength (663 mμ) which is absorbed by both chlorophylls a and b and at a wavelength (708 mμ) which is absorbed by the chlorophyll a pigment system but not by chlorophyll b (54).

The main results may be summarized as follows. At 708 mμ, i.e., at a wavelength at which light absorption by chlorophyll b was excluded, isolated chloroplasts were unable to use water as a hydrogen donor but retained the photoactivity which did not depend on water as a hydrogen (electron) donor. Thus, little oxygen evolution (Table 1 in ref. 12) was observed at 708 mμ (see also ref. 55), but at this wavelength chloroplasts were able to photoreduce ferredoxin and sustain a ferredoxin-catalyzed cyclic photophosphorylation. (Contrary to other reports (56) we found no cyclic photophosphorylation at 708 mμ with phenazine methosulfate.) At 708 mμ, chloroplasts were also able to sustain a noncyclic photophosphorylation coupled with TPN reduction but only when ascorbate-DPIP couple was supplied to replace water as the hydrogen donor system. The presence or absence of air had no special effect on either cyclic or noncyclic photophosphorylation at 708 mμ.

The photoactivity of isolated chloroplasts at 663 mμ differed from that at 708 mμ but was essentially the same as in white light. Both chlorophyll a and b were able to absorb light at this wavelength; water served as the electron donor for the photoproduction of ferredoxin, and the resulting reduction of TPN and noncyclic photophosphorylation was accompanied by oxygen evolution.

At 663 mμ, the presence or absence of oxygen had little effect on noncyclic photophosphorylation but had a marked effect on cyclic photophosphorylation catalyzed by ferredoxin. In the absence of oxygen, ferredoxin-catalyzed cyclic photophosphorylation occurred only when electron transport from water was blocked by the addition of CMU. No addition of CMU was required for a ferredoxin-catalyzed cyclic photophosphorylation at 663 mμ in air or, it will be recalled, under either aerobic or anaerobic conditions at 708 mμ. Thus the presence of oxygen was necessary for ferredoxin-catalyzed cyclic photophosphorylation only when the flow of electrons from water remained open; no oxygen was necessary when the electron flow from water was blocked, either

by the use of an inhibitor (CMU) at 663 mμ or by the use of far-red monochromatic light (708 mμ). A possible explanation of these effects of oxygen will be given later.

Roles of chlorophylls a and b in chloroplast electron transport. The results obtained with monochromatic light support and extend our earlier tentative conclusion (8) that the participation of chlorophyll b is essential for oxygen evolution but not for TPN reduction and ATP formation.

The diagram shown in Fig. 9 incorporates the new data into our 1961 scheme of electron transport in chloroplasts (Fig. 3 in ref. 7). We envisage that noncyclic photophosphorylation by chloroplasts is coupled with an "uphill" flow of electrons from water (OH^-) to TPN--an electron flow which is driven by two photochemical reactions working in series (cf. 57). The first photoreaction lifts electrons from the redox potential (at pH 7) of water-oxygen (E'_o = 0.82 V) to that of plastoquinone ($E'_o \sim 0$ V) and requires the participation of chlorophyll b (henceforth referred to as photoreaction B). The second photoreaction lifts electrons from the level of cytochrome f (E'_o = 0.365 V) to that of ferredoxin (E'_o = -0.43 V) and is driven by the chlorophyll a pigment system (henceforth referred to as photoreaction A). A "primary" phosphorylation site, common to both the cyclic and noncyclic photophosphorylation pathways, is considered to be coupled with a "downhill," dark electron transfer--one favored by the thermodynamic gradient--which joins the two photochemical reactions and probably involves a transfer of electrons from plastoquinone (Q) to the chloroplast cytochromes and thence to chlorophyll a (long black arrow in Fig. 9). In addition, the ferredoxin-catalyzed cyclic photophosphorylation is envisaged as having a least one more phosphorylation site, coupled with the electron transport sector that is marked in Fig. 9 by a broken line.

Role of oxygen in cyclic photophosphorylation. The differential oxygen effect on ferredoxin-catalyzed cyclic photophosphorylation at 663 and 708 mμ, mentioned previously, is explained by the following hypothesis. Noncyclic electron flow in chloroplasts is a unidirectional electron transfer from water to TPN and is driven by both photoreactions B and A. The problem of "overreduction" of intermediate electron carriers does not arise as long as the terminal electron acceptor, TPN, is available. A different situation, however, arises in the case of cyclic photophosphorylation. To maintain a cyclic electron flow from reduced ferredoxin back to the electron transport chain, the intermediates

in the chain must be at least partly oxidized. If they are kept in a reduced form they cannot accept electrons from reduced ferredoxin.

Our hypothesis proposes that molecular oxygen acts as a redox "buffer" for the electron transport chain involved in cyclic photophosphorylation by chloroplasts. In the presence of oxygen, the electrons from water cannot overreduce the electron carriers in the electron transport chain. Without this "buffering" effect of oxygen, the flow of electrons from water (through photoreaction B) overreduces the components of the electron transport chain in chloroplasts and the endogenous cyclic photophosphorylation via ferredoxin cannot proceed.

The hypothesis just presented is supported by experiments with two beams of light (13), as illustrated in Fig. 10. Fig. 10 shows that, under anaerobic conditions, cyclic photophosphorylation at 708 mμ is inhibited by the addition of a second monochromatic beam of light at 663 mμ. This chromatic inhibition, which we attribute to overreduction by the 663 mμ beam, occurred immediately when illumination by the combined 708 and 663 mμ beams was preceded by preillumination (under N_2) at 663 mμ (bottom curve, Fig. 10). Without an anaerobic preillumination treatment, the inhibitory effect of the 663 mμ beam, added to the 708 mμ beam, was observed only after 4 min. Evidently an interval of time was needed to bring about a sufficient state of overreduction by the 663 mμ beam.

Photochemical activity of subcellular preparations of blue-green algae. In blue-green algae, phycobilins and not chlorophyll b constitute the "accessory" pigment system for chlorophyll a. The phycobilin pigments are water soluble and can be readily separated from chlorophyll a. Thus, cell-free preparations of blue-green algae offer attractive possibilities for testing the view that the photoproduction of oxygen depends on the accessory pigment system and can be experimentally separated from photophosphorylation and TPN reduction.

Thomas and DeRover (58) have already reported that a loss of phycocyanin is associated with a loss of oxygen evolution by cell macerates of blue-green algae. Petrack and Lipmann (23) found that fragments of Anabaena cells which lost phycocyanin and the capacity for oxygen evolution still retained a capacity for cyclic photophosphorylation. Black et al. (59) showed that cell-free preparations of blue-green algae contain ferredoxin and are able to photoreduce TPN with either water or ascorbate-DPIP as

the electron donor system.

Mitsui and Arnon (60) have compared the photochemical activity of two kinds of particles, "blue" and "green", from Nostoc. The blue particles (prepared with carbowax (61) and dextrin) contained both chlorophyll a and phycocyanin whereas the green particles (prepared with carbowax only) contained little of the phycocyanin pigment.

Table 5

Noncyclic Electron Flow in Blue and Green Nostoc Chromatophores (60)

Electron donor system	Blue chromatophores		Green chromatophores	
	O_2 evolved	TPN reduced	O_2 evolved	TPN reduced
	μmoles		μmoles	
Water	3.0	7.6	0.4	0.9
Ascorbate-DPIP	0.9	7.2	0.2	6.2

As shown in Tables 5 and 6, both blue and green Nostoc particles were able to carry out cyclic photophosphorylation with phenazine methosulfate and photoreduce TPN with the ascorbate-DPIP couple as the electron donor system. However, the green particles had only a feeble capacity for TPN reduction (and oxygen evolution) when water was the electron donor. By contrast, the blue particles were able to use water effectively as the electron donor for a reduction of TPN and a coupled oxygen evolution.

Table 6

Cyclic Photophosphorylation in Blue and Green Nostoc Chromatophores (60)

	Blue chromatophores	Green chromatophores
	μmoles ATP formed	
Light	3.8	3.8
Dark	0.5	0.4

These results are consistent with the view that in particles of blue-green algae, as in chloroplasts, the accessory photosynthetic pigment is required for photoproduction of oxygen, but not for photophosphorylation and TPN reduction per se.

Concluding remarks. Extensive work from several laboratories, mainly with intact cells, has led to a now widely held view that photosynthesis in green plants involves the cooperation of at

least two pigment systems, each of which carries out separate partial reactions essential to the over-all process (see review, 62). Recent work with isolated chloroplasts, in which the partial reactions of photosynthesis have been experimentally separated, has contributed biochemical evidence in support of the idea of at least two collaborative photoreactions. Photoproduction of oxygen requires the participation of chlorophyll b but light absorption by this pigment is not essential for the photoreduction of ferredoxin and the ensuing TPN reduction and photophosphorylation. These seem to be associated with the chlorophyll a pigment system. Ferredoxin is assigned a key role in the energy conversion process in chloroplasts as an electron carrier in both cyclic and noncyclic photophosphorylation. A hypothesis is presented which attributes to molecular oxygen gas the function of a redox "buffer" in cyclic photophosphorylation by chloroplasts.

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Daniel I. Arnon

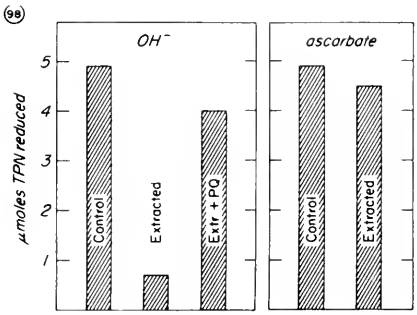

Fig. 1. Effect of extracting and restoring plastoquinone to spinach chloroplasts on photoproduction of TPN with either water (OH^-) or ascorbate as electron donor (18).

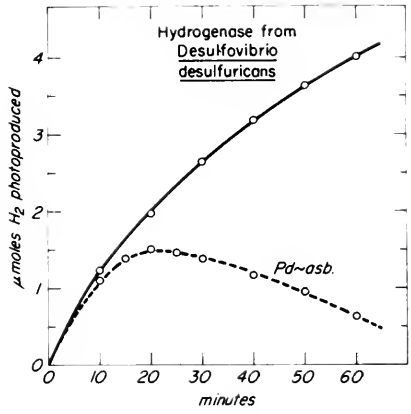

Fig. 2. Photoproduction of hydrogen gas by spinach chloroplasts supplemented by a hydrogenase from Desulfovibrio desulfuricans. H_2 was identified by adsorption on palladium asbestos (Pd-asb) (22,25).

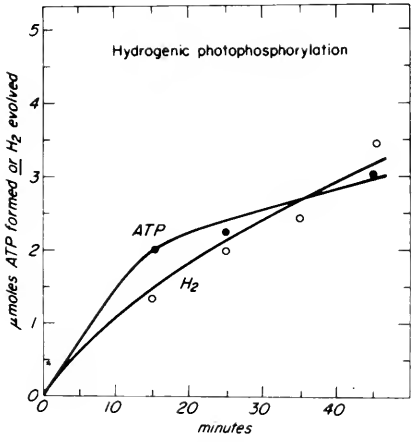

Fig. 3. Photoproduction of hydrogen coupled with photophosphorylation by spinach chloroplasts supplemented with a hydrogenase from Desulfovibrio desulfuricans (26,27).

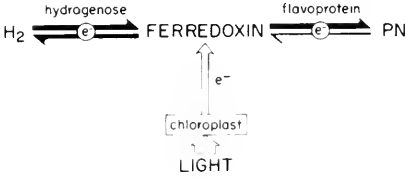

Fig. 5. Diagrammatic representation of the role of ferredoxin as an electron carrier (9).

Fig. 4. Microphotograph of crystalline ferredoxin-TPN reductase (30).

Fig. 6. Microphotograph of crystalline spinach ferredoxin (9).

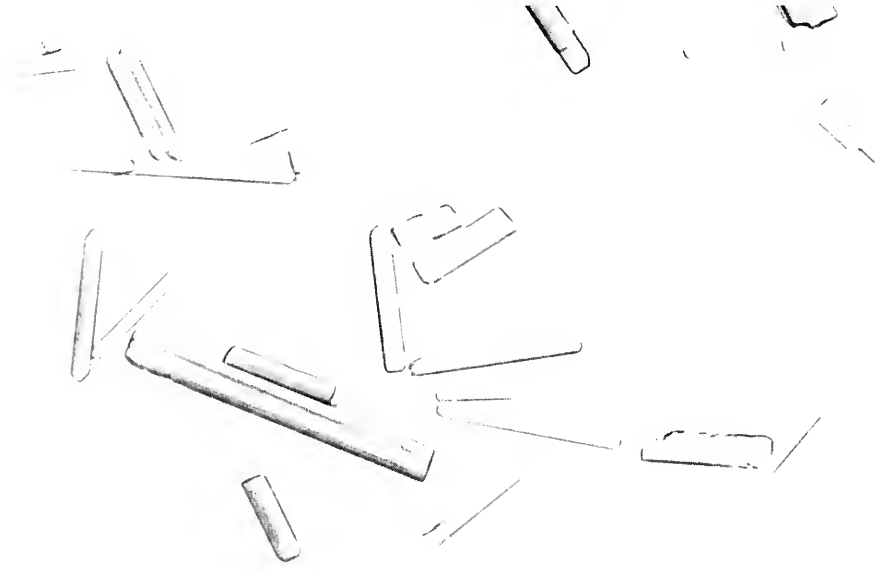

Fig. 7. Microphotograph of crystalline Chromatium ferredoxin (Bachofen, Oda and Arnon, 1963).

Fig. 8. Microphotograph of crystalline ferredoxin from Nostoc (60).

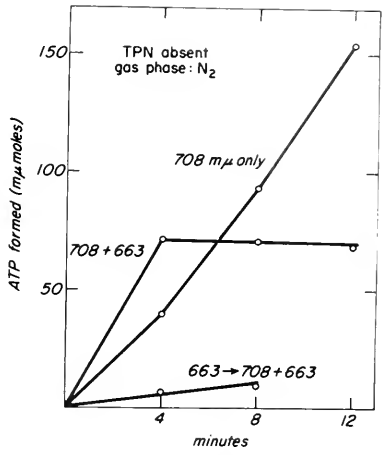

Fig. 10. Effect of additional illumination at 663 mμ on ferredoxin-dependent cyclic photophosphorylation at 708 mμ under anaerobic conditions (13).

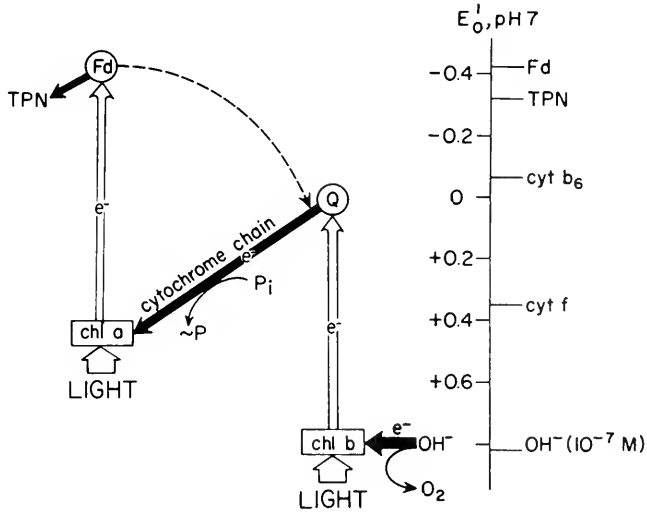

Fig. 9. Scheme for two photoreactions and their relation to cyclic and noncyclic photophosphorylation in chloroplasts (13).

CHARACTERISTICS OF TRITIUM INCORPORATION INTO ILLUMINATED CHLOROPLASTS

Brian Colman and Wolf Vishniac

It has been shown in previous experiments (1,2), that chloroplasts, incubated with tritium labeled water in the light, incorporate tritium into chlorophyll a. More tritium is incorporated in light than in the dark and little incorporation takes place with boiled chloroplasts. The tritium label of the chloro-phyll was found to be stable to acid but unstable to alkali, which suggested that the chlorophyll was labeled at the C-10 position.

Quantitative evaluation of the data was rendered difficult by the unexplained loss of radioactivity from samples of purified chlorophyll a stored in organic solvents at -15°. This loss has been found to be much more rapid when samples are dried prior to counting in a windowless gas flow counter, and makes it impossible to follow the kinetics of photosynthetic incorporation. In order to minimize the loss of activity, samples were placed in the counter while still in solution and allowed to dry in the stream of counting gas. By this procedure the rate at which radio-activity dissappeared from labeled chlorophyll could be followed. Labeled chlorophyll a was obtained from chloroplasts which had been illuminated in suspension together with tritiated water. The chloroplasts were extracted with acetone, the acetone fraction transferred to heptane, and chlorophyll a rapidly separated from the other pigments by chromatography on thin layers of powdered sugar. (Sucrose is applied to glass plates as a suspension in methanol; the small amount of sucrose that dissolves in the meth-anol serves to bind the remainder to the plate). Figure 1, curve 2, shows the loss of radioactivity from labeled chlorophyll a isolated from chloroplasts. Curve 1. illustrates a similar decay, but here the chlorophyll was labeled chemically. Purified chloro-phyll a in ether was treated with tritiated water and 2% pyridine at laboratory temperature. The enol-keto tautomerization resulting from this treatment with base, presumably leads to the replacement of the protium on C-10 with tritium.

Brian Colman and Wolf Vishniac

Chlorophyll a treated with tritiated water under neutral conditions, that is in boiling ether, incorporates only a fraction of the activity of that treated under basic conditions and the resultant label is relatively stable (Fig.2, curve 2, cf. curve 1.). Phaeophytin a labeled by each of the two procedures shows the same types of decay as chlorophyll a. During the loss of radioactivity there is no chemical change in the chlorophyll or phaeophytin as judged by spectroscopic observation. Allomerized chlorophyll a, prepared by the oxidation of chlorophyll a in methanol with gaseous oxygen, was treated with tritiated water in 2% pyridine in ether. The small amount of radioactivity incorporated and its stability (Fig.2, curve 3) are consistent with the notion that the tritium label of the intact chlorophyll a is located on C-10. Heating allomerized chlorophyll a in ether in the presence of TOH did not lead to significant labeling either.

The loss of radioactivity from chlorophyll does not reflect evaporation of tritiated water originally introduced into the reaction mixture. The procedure for the isolation of the chlorophyll precludes the retention of any such water. Instead the disappearance of tritium suggests that it is either given off as water vapor or as hydrogen gas resulting from an unknown exchange reaction.

The light-dependant incorporation of tritium into chloroplast components is affected by CMU (p-chlorophenyl-1,1-dimethyl urea). CMU has been reported (3,4) to inhibit the Hill reaction, the reduction of TPN and photosynthetic phosphorylation in chloroplast preparations. Pea chloroplasts were incubated with tritiated water under the conditions described in Table 1. At the end of the experiment, the chloroplasts were extracted with acetone and the acetone fraction transferred to heptane. The residue of the chloro-plasts was then succesively extracted with isooctane, methanol and water. The data in Table 1. confirm the earlier results that more activity is taken up by the component pigments of the heptane fraction in the light than in the dark, and also show that this light stimulated uptake is inhibited by CMU.

The accumulation of tritium in the isooctane fraction in the presence of CMU is interesting. This fraction contains at least two quinones which incorporate tritium from water in the light. While tritium incorporated into a quinone would be expected to be readily exchangeable with water, it had been suggested to us by Dr. Calvin that the retained activity could result from the rearrangement of a suitable reduced quinone, for example, reduced Co Q, to form a chroman ring with the concomitant migration of

the hydroxyl tritium to the side chain. Upon reoxidation the label would therefore be located on the phytol side chain. In a model experiment, purified Co Q from Chromatium was reduced with $NaBH_4$ in the presence of TOH, reoxidised with silver oxide, and reisolated. The retention of tritium by the Co Q to the extent of half the specific activity of the TOH, confirmed the possibility of labeling the quinone by the mechanism which Dr. Calvin had proposed.

The methanol fraction contains six, and the water fraction two chromatographically separable, but unidentified compounds, all of which incorporate more tritium in the light than in the dark.

Although we cannot explain the accumulation of tritium in the isooctane fraction, the inhibition by CMU of tritium incorporation into photosynthetic pigments is a further indication that tritium can serve as a useful tracer in the investigation of photosynthetic events.

Table 1. Incorporation of tritium by chloroplasts in the presence of CMU.

Fraction	Dark		Light	
	No CMU	+CMU	No CMU	+CMU
Heptane	13,840	16,400	84,400	10,800
Isooctane	9,450	18,350	17,700	349,000
Methanol	165,000	150,500	358,000	417,000
Water	9,350	15,550	16,300	16,400

Figures in c.p.m. The reaction mixture contained chloroplasts (5 mg chlorophyll) in sucrose-potassium phosphate, pH 7, 100 mC H^3OH, and CMU (10^{-3}M) where indicated. Total volume 1.0 ml. Temperature 10°C.

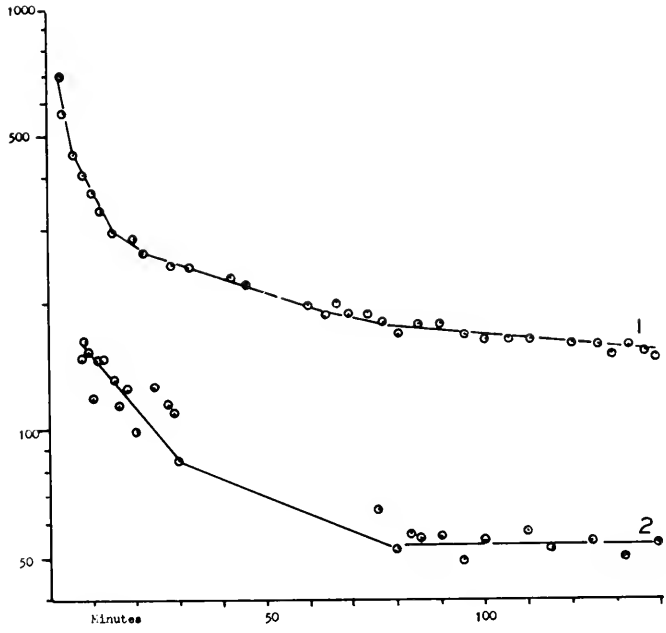

Figure 1. Loss of radioactivity from dry chlorophyll samples.
Curve 1: Chlorophyll a treated with H^3OH in ether plus 2% pyridine.
Curve 2: Chlorophyll a isolated from chloroplasts illuminated in presence of H^3OH.
Ordinate in c.p.m. per sample counted.

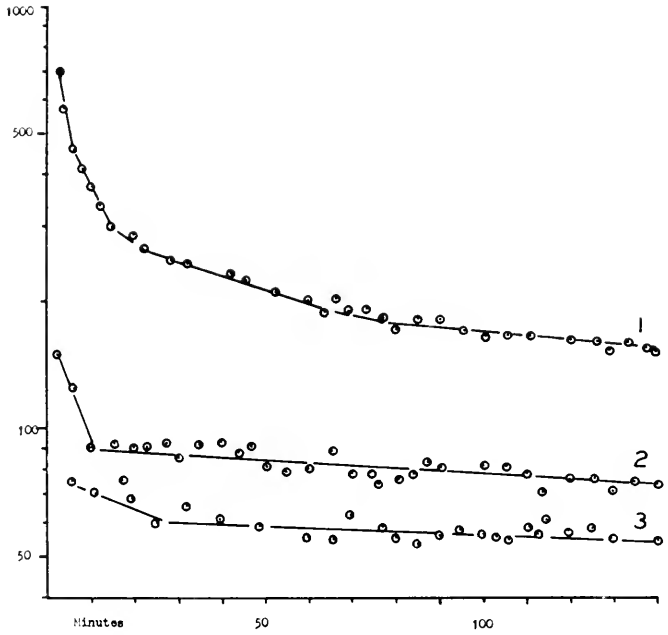

Figure 2. Characteristics of tritium labeling of chlorophyll a and allomerized chlorophyll a.
Curve 1: Purified chlorophyll a treated with H^3OH in ether + 2% pyridine.
Curve 2: Purified chlorophyll a boiled in ether with H^3OH
Curve 3: Allomerized chlorophyll a treated with H^3OH in ether + 2% pyridine.
Ordinate in c.p.m. per sample counted.

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INDOPHENOL DYES: CATALYSTS AND UNCOUPLERS OF PHOTOPHOSPHORYLATION

Donald L. Keister

The indophenol dyes, especially 2,6-dichloroindophenol (DCI) and 2,3',6-trichloroindophenol (TCI) have been known to be excellent oxidants for measuring the Hill reaction since 1948[1]. In 1954[2], the demonstration was made that isolated chloroplasts under the influence of light could phosphorylate ADP and in 1958[3] it was discovered that the reduction of TPN was accompanied by ATP formation. Subsequently it has been shown that ATP formation was coupled to the reduction of other Hill oxidants including ferricyanide, FMN and cytochrome c. However, it has been reported in the literature that the reduction of the indophenol dyes was not accompanied by photophosphorylation[4,5]. This view has led to the postulation that these compounds were reduced by some component of the electron transport chain prior to the site of phosphorylation.

Earlier this year three widely separated groups, Shen et al.[6] in China, Gromet-Elhanan and Avron[7] in Israel, and our own[8], independently discovered that phosphorylation was coupled to the reduction of indophenols. These compounds are potent uncouplers of photophosphorylation in their oxidized form and therein lies the reason that the phosphorylation coupled to their reduction was previously overlooked.

METHODS

Unless otherwise specified each reaction mixture per 3 ml contained the following in μmoles: Tris, 150; ADP, 2; $MgCl_2$, 5; ^{32}Pi, 2-10; and chlorophyll as noted. After illumination the reactions were terminated by the addition of 0.3 ml of 50% trichloroacetic acid (TCA) and uptake of ^{32}Pi determined as previously noted[8]. Anaerobic experiments were performed in Warburg flasks or in 50 ml Erlenmeyer flasks fitted for flushing with gas. The flasks were flushed with a strong stream of argon and shaken throughout the experiment. Ten minutes of flushing was found sufficient to remove essentially all the oxygen. Where reduced DCI or TCI was added, it was reduced with hydrogen using platinum asbestos as catalyst thus eliminating any other reducing agents. One drop of bromine water was added to samples for ^{32}Pi uptake determinations after the addition of TCA to oxidize the reduced indophenols and prevent their interference with the determination. Cytochrome c was the Type III preparation of the Sigma Chemical Company.

Donald L. Keister

PHOSPHORYLATION COUPLED TO THE REDUCTION OF INDOPHENOLS

The phosphorylation coupled to the reduction of the indophenol dyes was demonstrated by all three of the above mentioned groups by using very low concentrations of dye and measuring the small amount of ATP formed by the incorporation of high specific activity ^{32}Pi into ATP during short illumination periods. Gromet-Elhanan and Avron[7] using 5 x 10^{-6}M DCI and short illumination periods measured ATP formation at rates up to 163 µmoles/mg chlorophyll/hr. They further demonstrated that the reduction of DCI was stimulated by the inclusion of a phosphate acceptor system (ADP, Mg^{++}, and Pi) or by ammonium chloride, a well-known uncoupler of photophosphorylation. These criteria place this compound in the same category as ferricyanide[9] and TPN[10] as Hill oxidants.

Shen et al.[6] using essentially the same techniques but with higher light intensities demonstrated that rates of ATP formation up to 640 µmoles/mg chlorophyll/hr could be obtained with 1.28 x 10^{-5}M DCI with an ATP:$2e^-$ ratio of 1.0. This is the highest rate yet reported for a non-cyclic phosphorylation.

Keister[8] first noted that TCI would catalyze ATP formation while studying the photoreduction of cytochrome c by chloroplasts[11]. This is illustrated in Fig. 1.

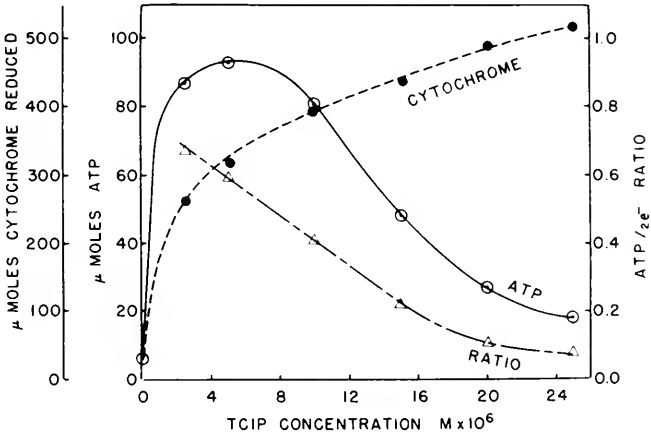


Fig. 1. The effect of TCI concentration on phosphorylation coupled to cytochrome c reduction. The reaction mixture, pH 7.5, contained 3 µmoles KCN, 3.8 mg cytochrome c, and 10 µg chlorophyll in addition to those described in methods. Illumination was for 2 min with 5000 ft. candles of light.

In this system TCI was reduced photochemically and oxidized by cytochrome c. Thus, the TCI remains primarily in the oxidized

form. Under these conditions ATP formation was observed to accompany cytochrome c reduction with an ATP:$2e^-$ ratio that approached 1.0 at low TCI concentrations (3×10^{-6}M).

INDOPHENOLS AS UNCOUPLERS OF PHOTOPHOSPHORYLATION

Fig. 1 also illustrates the uncoupling effect of TCI. As the TCI concentration was increased the rate of cytochrome c reduction was stimulated while ATP formation was inhibited until at 2.5×10^{-5}M TCI, very little ATP synthesis occurred. This clearly demonstrates the uncoupling effect of the oxidized indophenols. A further demonstration of this uncoupling effect is illustrated in Fig. 2 which shows the inhibition of cyclic photophosphorylation by TCI. This experiment was performed under argon in the presence of 10^{-5}M p-chlorophenyl-1,1-dimethylurea (CMU) to inhibit dye reduction and thus maintain the TCI in its oxidized form. Under these conditions, TCI increasingly inhibits cyclic phosphorylation with almost complete inhibition by 10^{-5}M TCI. The curve labelled "reduced TCI" (Fig. 2) demonstrates that the reduced form of the dye has no inhibiting effect. Avron and Jagendorf[12] first observed the inhibition of cyclic phosphorylation by TCI but at that time concluded that the dye was interfering with electron transport reactions rather than uncoupling or inhibiting phosphate transfers.

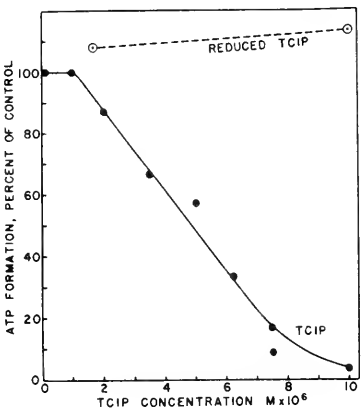
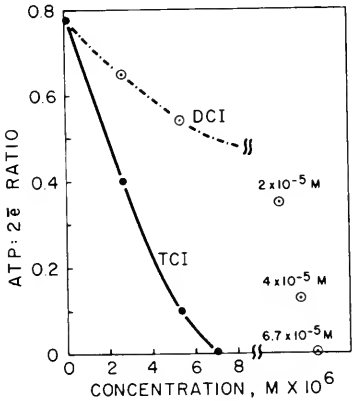


Fig. 2. The inhibition of cyclic phosphorylation by TCI. The reaction mixture, pH 8.2, contained 0.06 µmole pyocyanin, 0.03 µmole CMU, and 22 µg chlorophyll in addition to those described. The reaction was run in Warburg vessels with pyocyanin, TCI, and CMU being tipped in after flushing for 10 min with argon. The control rate of phosphorylation during a 6 min illumination period with pyocyanin alone was 214 µmoles/mg/hr which was inhibited 20% by CMU.

Shen et al.[6] have also concluded that the indophenols are uncouplers of phosphorylation from two types of experiments. They have previously shown that ATP formation and light-induced electron transport could be separated by pre-illuminating chloroplasts in the presence of phenazine methosulfate (PMS) but without ADP and Pi. After a short but intense illumination, the addition of ADP and ^{32}Pi results in the formation of ATP[13]. When increasing concentrations of DCI was added along with the ADP and Pi, the ATP formation was inhibited up to 85% by 10^{-4}M DCI while the reduced dye had no effect. This type of inhibition was interpreted as uncoupling. In addition they have measured ATP formation and electron transport by chloroplasts in a system containing both DCI and ferricyanide. With only ferricyanide present an ATP:$2e^-$ ratio of 1.26 was measured. With increasing concentrations of DCI the ratio decreased until complete inhibition of ATP formation was observed with 10^{-4}M DCI. Ferricyanide reduction was stimulated 25% by this concentration of dye.

Fig. 3 illustrates this uncoupling effect of both DCI and TCI on ferricyanide coupled photophosphorylation. Complete uncoupling was observed with 7 x 10^{-6}M TCI while almost a ten-fold higher concentration of DCI was required for complete uncoupling. 3'-chloroindophenol was also shown to be an uncoupler at higher concentrations.



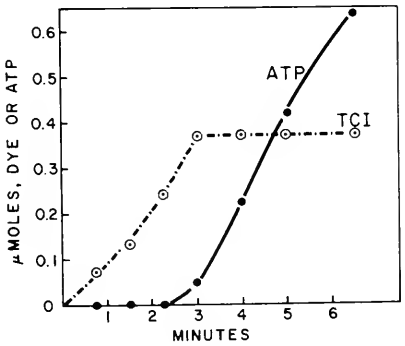


Fig. 3. (Left) The uncoupling effect of indophenols on ferricyanide coupled phosphorylation. The reaction mixture, pH 8.0, contained 2 μmoles of ferricyanide and 100 μg chlorophyll in addition to those described in methods.
Fig. 4. (Right) The time course of TCI reduction and ATP formation. The reaction mixture, pH 8.0, contained 1.23 x 10^{-4}M TCI and 112 μg chlorophyll in addition to those described in methods per 3 ml of solution. One ml samples were removed for measurement of ^{32}Pi uptake and TCI reduction.

Losada et al.[14] first reported that when indophenols were added to a reaction containing ferricyanide the rate of oxygen evolution was accelerated, whereas the coupled phosphorylation was inhibited. Since they also found that there was no inhibition of ATP formation coupled to TPN reduction by the same concentrations of dye (in fact there was a stimulation), they proposed that the indophenols were the direct electron acceptors, not coupled to phosphorylation, which were reduced at a point in the electron transport chain prior to the site of phosphorylation. In the TPN system the indophenols were rapidly reduced and not reoxidized by TPN (the reduced dyes are not uncouplers). Also, oxygen evolution can be blocked by CMU, and ATP formation and TPN reduction restored by reduced dye and ascorbate[15], thus eliminating the requirement for the photooxidation of water. On the basis of these observations they proposed a separation of the photooxidation of water and non-cyclic phosphorylation into two distinct photochemical reactions. Although it appears that these are indeed two separate reactions, the use of the indophenol dyes to separate them was not valid since the above results demonstrated that there was ATP formation coupled to their reduction and that they are potent uncouplers.

Shen et al.[6] have even cast some doubt on whether in a system containing both ferricyanide and indophenol, that the dye is the direct electron acceptor. Taking advantage of the differential inhibition of DCI and ferricyanide reduction by hydroxyquinoline N-oxide[16] (HOQNO), they demonstrated that in a system containing both ferricyanide and DCI, the degree of inhibition by HOQNO corresponded to the degree of inhibition observed with ferricyanide alone and not to that observed with DCI alone. Thus it appears that in the DCI-ferricyanide system, ferricyanide is the direct electron acceptor and the DCI an inert uncoupler.

CYCLIC PHOTOPHOSPHORYLATION

The ability of reduced indophenols to reduce a photochemical oxidant and thus restore the reduction processes in chloroplasts, in which the normal process has been blocked by inhibiting oxygen evolution either with CMU or aging[15], has been established. From these results it could have been postulated that the reduced indophenols should catalyze a cyclic electron transport since they can be both oxidized and reduced by chloroplasts. This was first demonstrated by Trebst and Eck[17] who reported that reduced DCI and TCI catalyzed a phosphorylation by chloroplasts that was not inhibited by DCMU. They interpreted this as a cyclic phosphorylation. These results were later confirmed by three laboratories[6-8].

Krogmann and Vennesland[5] earlier had reported that the indophenol dyes could mediate an oxygen dependent photophosphorylation and suggested that the phosphorylation occurred upon the photooxidation of the dye and not during the reduction. This suggestion was based upon: 1) reduced dyes catalyzed the ATP formation; 2) the phosphorylation was inhibited by a nitrogen atmosphere; and 3) DCMU inhibition could be reversed by reducing

the indophenol prior to illumination. The observations can all be explained by cyclic phosphorylation.

Fig. 4 illustrates a time course of the reduction of TCI and ATP formation under aerobic conditions. There was no ATP formation in this experiment until the reduction of dye had almost reached completion. The rate of phosphorylation was the greatest just after the completion of dye reduction and was proportional to time thereafter. The ATP formation in Krogmann and Vennesland's experiments was measured only at a single time interval, and thus the inhibition of ATP formation before the dye was reduced was overlooked.

The concentration dependence of phosphorylation with DCI and TCI is shown in Fig. 5. Approximately a 3-fold higher concentration was required for optimum ATP formation under argon than with an air atmosphere. It is to be noted that concentrations optimal for aerobic phosphorylation catalyze very little anaerobic phosphorylation and thus would appear to be inhibited by removing oxygen. One explanation for the different optimal concentrations is that the reoxidation of the dye by the chloroplast is the rate limiting step in the anaerobic system and the active site for the oxidation has a lower affinity for the dye than does the active site for the reduction. Since the autooxidation of the indophenols by oxygen is rather marked at alkaline pH's, this source of oxidized dye would enhance the turnover of dye in the aerobic system thus shifting the optimal concentration toward the lower optimal concentration of the reducing system. Therefore, a lower concentration would be required for the aerobic phosphorylation.

The effect of light intensity on ATP formation with reduced indophenols as compared with pyocyanin and FMN is illustrated in Fig. 6. The FMN and pyocyanin curves are identical for both the aerobic and anaerobic systems, therefore, only one curve was drawn for each. The FMN system became saturated at fairly low light intensities as has been previously reported for FMN[18] and TPNH[19] which catalyze non-cyclic phosphorylation, whereas the pyocyanin which catalyzes cyclic phosphorylation did not saturate even at very high light intensities[20]. Fig. 6 demonstrates that under aerobic conditions with reduced DCI as catalyst of photophosphorylation the reaction became saturated at low light intensities similar to that of FMN, whereas under argon the curve is similar to that of pyocyanin which was not saturated with light. These results are indicative that anaerobically the indophenols catalyze a cyclic phosphorylation similar to that of pyocyanin, whereas with oxygen present the reaction appears to be of the non-cyclic type. However, in view of other results it is more probable that it is a combination of cyclic and non-cyclic phosphorylation.

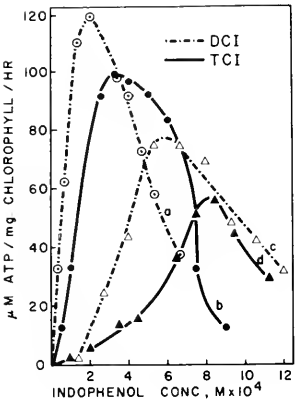


Fig. 5

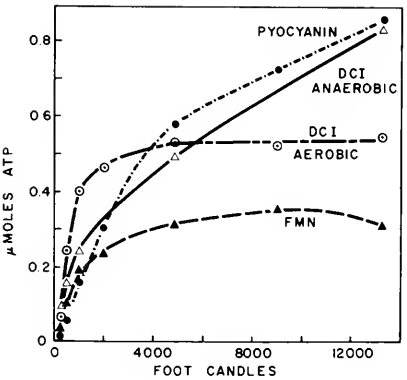


Fig. 6

Fig. 5. The effect of reduced indophenol concentration on aerobic and anaerobic phosphorylation. The reaction was illuminated in 50 ml Erlenmeyer flasks fitted for flushing with gas. Illumination was provided by 75 watt photoflood lamps which provided 5400 ft. candles. Curves a and b, gas phase is air. Curves c and d, gas phase is argon.

Fig. 6. The effect of light intensity on phosphorylation catalyzed by DCI, pyocyanin, and FMN. The reaction mixture contained either 2.6 x 10^{-4}M DCI, 10^{-4}M FMN, or 10^{-5}M pyocyanin and 80 μg chlorophyll in addition to those reagents described in methods. The reaction was illuminated from underneath in Warburg vessels. The light intensity was varied by changing the distance of the light source from the flask and by inserting wire screens. Anaerobic flasks were flushed for 10 min with argon before illumination. Illumination time was varied so that curves were comparable.

CONCLUSIONS

The indophenol dyes have now been shown to catalyze two types of photophosphorylation; a non-cyclic phosphorylation coupled to their reduction, thus placing them in the same category as ferricyanide as a Hill oxidant; and a cyclic photophosphorylation similar to that catalyzed by PMS and pyocyanin. In addition they act as a mediator to supply electrons from ascorbate and thus restore TPN reduction by a system in which oxygen evolution has been blocked or destroyed[15]. It appears probable that this site of action and the site where the dye is reoxidized during cyclic phosphorylation are the same.

The indophenols were also demonstrated to be potent uncouplers of photophosphorylation at concentrations normally used to

measure the Hill reaction. It is this property of the dyes which led previous investigators to believe that there was no ATP formation coupled to their reduction and thus postulate by necessity that the dyes intercept electrons at some point in the electron transport chain prior to the site of phosphorylation.

The uncoupling action of the dyes probably accounts for the observations of Whittingham and Bishop[21], who recently observed that the time between flashes of light was considerably shorter for optimum production of oxygen with DCI than with ferricyanide as the electron acceptor. They suggested, therefore, that production of oxygen by reduction of dye did not proceed through the same thermal reaction (phosphorylation) required for ferricyanide reduction. They also found that if the ferricyanide reduction was uncoupled from phosphorylation by ammonium ions, the optimum time between flashes was of the same magnitude as with DCI. Witt and coworkers[22] observed that DCI accelerates the decay of "Type 2" absorption increases at 515 mμ in chloroplasts. This decay is a thermochemical process, and it is likely that the acceleration of decay can now be explained by the uncoupling effect of the indophenols.

It is of interest that Löw *et al.*[23] have reported that oxidized but not reduced DCI inhibited Pi-ATP exchange reactions in rat liver mitochondria.

Hill and Walker[24] several years ago observed that, "The phosphorylation reaction itself now appears as a part of normal photochemically induced H-transfer." They also observed,

> "It does not follow...that all substances capable of being reduced by illuminated chloroplasts preparations would be capable of initiating phosphorylation. The coupling between reduction and phosphorylation can be abolished and an active agent can become inhibitory at higher concentrations so that affinities relating to the chloroplast system have to be in a suitable range."

Contribution No. 134 of the Charles F. Kettering Research Laboratory.

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PHOTOSYNTHETIC PHOSPHORYLATION IN THE PRESENCE OF NATURALLY OCCURRING SUBSTANCES

C. C. Black and A. San Pietro

After the initial observation of photosynthetic phosphorylation [1, 2] it was demonstrated that many common laboratory redox chemicals support photophosphorylation. A list of these chemicals would include: methyl phenazonium methosulfate, or pyocyanine [3-5]; ferricyanide [6]; viologen dyes [5, 7, 8]; anthraquinone [5]; dimethyl safranin sulphonate [5]; methylene blue [5]; indigo carmine [7]; and indophenol dyes [9-12]. The natural occurrence, hence physiological role, of these chemicals is doubtful. The following substances occur naturally, may have a role of physiological importance, and have been shown to support photophosphorylation: vitamin K [13]; FMN [14]; riboflavin [14]; NADP [6,15]; plastoquinone [16]; allagochrome [17]; a "flavone-type" compound [18]; cytochrome c [19]; and photosynthetic pyridine nucleotide reductase (PPNR)[20]. Although these substances occur in nature and may play a physiological role, it appears that they are involved in the electron transfer pathway rather than the phosphorylation process.

Since scant experimental data was available concerning the components involved in the phosphorylation mechanism, research was undertaken to isolate a naturally occurring substance or substances involved in photophosphorylation. Evidence will be presented which indicates that we have successfully obtained a new naturally occurring catalyst of photosynthetic phosphorylation. The catalyst(s) has been detected in all types of photosynthetic organisms, and the catalyst from one organism has been shown to initiate photophosphorylation with both chloroplast and chromatophore fragments. We have tentatively assigned the name phosphodoxin to the catalyst.

ISOLATION AND DISTRIBUTION

Acetone powders of intact spinach chloroplasts were the first source of phosphodoxin [21]. We soon learned that phosphodoxin was not destroyed by heating at 100° C for periods up to 30 minutes; therefore, whole leaves or whole cells extracted with boiling water proved to be convenient sources of phosphodoxin. Most of the work in this paper will deal with spinach phosphodoxin since it has been studied more exhaustively than phosphodoxin from other photosynthetic organisms. More extensive data relating to the activity of phosphodoxin isolated from photosynthetic bacteria can be obtained in references 21 and 22. A partial list of the photosynthetic organisms in which phosphodoxin has been detected is given in Table 1, along with the

μmoles of ATP produced. Phosphodoxin appears to be ubiquitous among photosynthetic organisms.

Table 1

Photosynthetic Organisms Containing Phosphodoxin

Source	μmoles of ATP/mg chlorophyll/hr.*
Spinach, chloroplasts	196
Spinach, leaves	220
Phormidium luridum, whole cells	49
Tribonema aequale, whole cells	27
Euglena gracilis, whole cells	25
Chlorella pyrenoidosa, whole cells	210
Rhodospirillum rubrum, chromatophores	54
Rhodospirillum rubrum, whole cells	50
Chromatium, strain D, chromatophores	56
Chromatium, strain D, whole cells	115

*All preparations assayed with spinach chloroplasts

Spinach phosphodoxin was purified by acetone fractionation and paper chromatography [21]. Absorption spectra of spinach phosphodoxin is given in Fig. 1. The absorption spectrum shifts with pH, in contrast to the fluorescence activation spectra, which does not show a pronounced shift with pH (Fig. 2). Since the intensity of the fluorescence spectrum is pH-dependent but the activation spectrum is not (Fig. 2), it appears that the alkaline form of spinach phosphodoxin may be the fluorescent type. Alkaline solutions of spinach phosphodoxin are yellow, while acid solutions are nearly colorless. The fluorescent maximum was at 440 mμ and the activation maximum was at 358 mμ.

Aqueous solutions of spinach phosphodoxin are stable at 4° C when stored near neutrality. Boiling phosphodoxin for 10 minutes in N HCl does not affect its activity, whereas boiling in N NaOH inactivates the phosphodoxin. Irradiation by ultraviolet light does not alter its activity. Spinach phosphodoxin does not contain a functional metal [23]. At the present stage of our research, we do not know the structure of phosphodoxin, nor are we certain that each photosynthetic organism contains the same compound.

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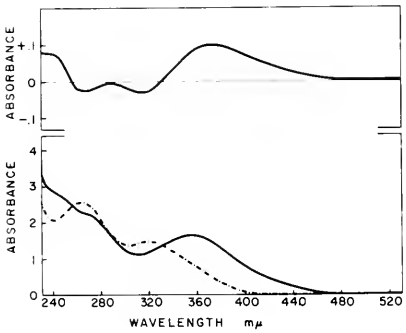


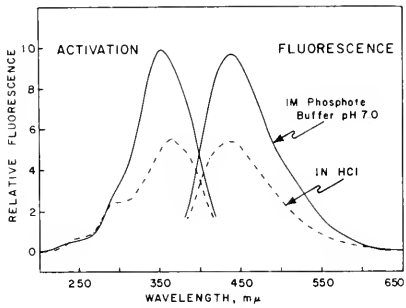


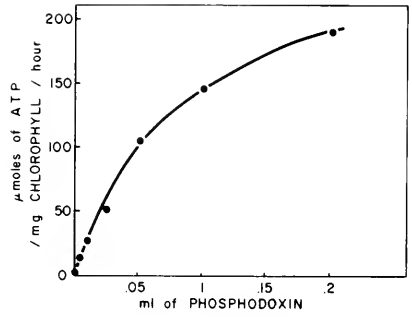
Fig. 1. (Left) Lower portion: absorption spectra of spinach phosphodoxin. Solid line, 0.1 N NaOH; broken line, 0.1 N HCl. Upper portion: difference spectrum.
Fig. 2. (Right) Activation and fluorescence spectra of spinach phosphodoxin.

BIOLOGICAL CHARACTERISTICS

The endogenous spinach chloroplast fragment photophosphorylation (0.5 to 3 umoles of ATP/mg chlorophyll/hr) is stimulated over 200-fold by the addition of spinach phosphodoxin (Fig. 3). As indicated in Fig. 4, this aerobic reaction is linear for short periods of illumination.

In an earlier publication [21] the reaction was only 60 per cent inhibited under nitrogen. Further work with prepurified nitrogen indicates that the reaction with spinach chloroplast fragments definitely requires aerobic conditions (Fig. 4). It should be noted that this is in contrast to the photophosphorylation catalyzed by Rhodospirillum rubrum phosphodoxin with R. rubrum chromatophore fragments, which is unaffected by anaerobiosis[21,22].

Photophosphorylation with spinach chloroplast fragments plus spinach phosphodoxin has been shown: to be linear with chlorophyll up to 40 μgrams of chlorophyll per ml; to have a pH optimum in Tris-HCl buffer between 7.4 and 7.8; to require a divalent ion for maximum activity; to respond in a sigmoid fashion to increasing intensity of white light with a distinct lag up to 100 foot-candles, reaching saturation between 1000 and 2000 foot-candles; and to be unaffected by PPNR, pyridine nucleotide transhydrogenase, and the antibody to the transhydrogenase. From Fig. 5 it can be seen that photophosphorylation catalyzed by spinach phosphodoxin is sensitive to the usual inhibitors of photophosphorylation. In addition to these inhibitors 50%



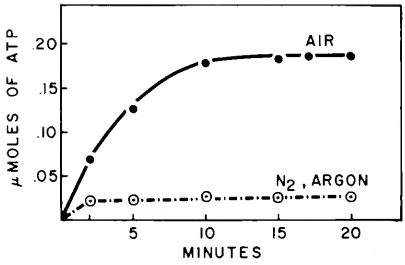


Fig. 3. (Left) Effect of concentrations of spinach phosphodoxin on photophosphorylation with spinach chloroplast fragments.
Fig. 4. (Right) Time course and effect of anaerobic conditions on photophosphorylation with spinach chloroplast fragments plus spinach phosphodoxin.

inhibition was obtained with the following compounds at the indicated concentrations: atebrin, 10^{-5} M; antimycin A, 4×10^{-5} M; Cd^{++}, 10^{-4} M; and NH_4^+, 6×10^{-4} M. Arsenite at concentrations as high as 10^{-3} M did not affect photophosphorylation with spinach phosphodoxin.

Since phosphodoxin is a naturally occurring catalyst, it was of interest to study photophosphorylation in the presence of other known catalysts. Total photophosphorylation in the presence of NADP and PPNR plus increasing amounts of spinach phosphodoxin did not change from that observed with only NADP and PPNR. In the presence of ferricyanide, a definite inhibition of ATP production was observed. A distinct stimulation of PMS-catalyzed photophosphorylation was observed, varying between 2- and 4-fold at low levels of phosphodoxin.

Spinach phosphodoxin is active with chloroplasts from higher plants and chromatophores from photosynthetic bacteria. Conversely, phosphodoxin from photosynthetic bacteria is active with spinach chloroplast fragments. This crossing of activity irrespective of the photosynthetic organisms from which the phosphodoxin was isolated is illustrated in Table 1 and in Fig. 7. Further demonstrations of this crossing of activity are given in references 21 and 22.

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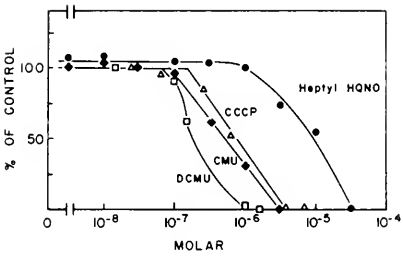


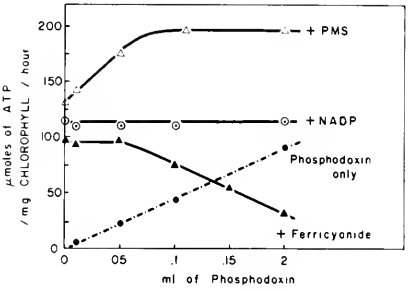
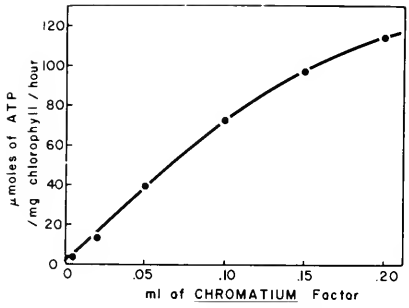


Fig. 5. (Left) Effect of inhibitors on photophosphorylation with spinach chloroplast fragments plus spinach phosphodoxin.
Fig. 6. (Right) Effect of spinach phosphodoxin on photophosphorylation with spinach chloroplast fragments in the presence of other electron acceptors.

ELECTRON PARAMAGNETIC RESONANCE SIGNAL

The EPR signals observed with spinach phosphodoxin have been studied in cooperation with Dr. John Heise [24]. Aqueous solutions of spinach phosphodoxin exhibit a light-induced, pH-dependent EPR signal. The intensity of the EPR signal is increased with alkaline conditions in a fashion similar to the relative fluorescence intensity (Fig. 2). No dark EPR signal is observed with spinach phosphodoxin alone and the light-induced signal decays in the dark.

Figure 8 demonstrates the effects of spinach phosphodoxin on the EPR signal of spinach chloroplast fragments in 4.2×10^{-2} M Tris-HCl buffer, pH 7.8. The small characteristic light-induced EPR signal of spinach chloroplast fragments alone [25] can be observed by comparing curves 1 and 4 of Fig. 8. A sharp decrease in the dark EPR signal of spinach chloroplast fragments upon the addition of spinach phosphodoxin can be observed by comparing curves 1 and 2 of Fig. 8. Upon illumination of spinach chloroplast fragments plus spinach phosphodoxin, an increased EPR signal was observed (compare curves 3 and 4 of Fig. 8). This increased EPR signal was observed immediately upon illumination and decayed with continuous illumination. Examination of the data indicates that spinach phosphodoxin contributes primarily to signal 2 [25] of spinach chloroplast fragments [24].



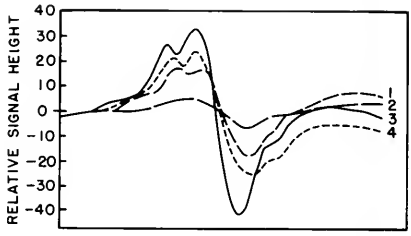


Fig. 7. (Left) Effect of Chromatium phosphodoxin on photophosphorylation with spinach chloroplast fragments.
Fig. 8. (Right) Effect of spinach phosphodoxin on EPR signals of spinach chloroplast fragments. Curve 1 - Dark; chloroplasts alone. Curve 2 - Dark; chloroplasts plus phosphodoxin. Curve 3 - Light; chloroplasts plus phosphodoxin. Curve 4 - Light; chloroplasts alone.

In summary, we have isolated a new naturally occurring, thermostable, water-soluble catalyst (phosphodoxin) of photophosphorylation which appears to be ubiquitous among photosynthetic organisms. Phosphodoxin from each photosynthetic organism appears to be active with both chloroplast and chromatophore fragments. Spinach phosphodoxin alone has a light-induced EPR signal and in combination with spinach chloroplast fragments decreases the dark EPR signal and increases the light EPR signal, and contributes primarily to signal 2.

This is Contribution No. 129 from the Charles F. Kettering Research Laboratory.

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CHARACTERIZATION OF ALLAGOCHROME AND ITS BIOSYNTHESIS IN LEAF EXTRACTS

Helen M. Habermann

Allagochrome is a blue-green pigment present in alkaline homogenates of a wide variety of plant species. Other colored substances (including at least two yellow fractions) are normally present in crude allagochrome preparations. A survey of the distribution of allagochrome in higher plant groups and a description of techniques which have been developed for the purification and assay of allagochrome have been published [2,5]. There is at least circumstantial evidence that allagochrome is involved in photophosphorylation and respiration [2,4,6].

The purpose of this paper is to summarize the recent progress toward understanding the structure and biosynthesis of allagochrome. Until recently there were few clues about the chemical nature of this pigment and at times the available information was misleading.

ALLAGOCHROME BIOSYNTHESIS

For the past several months we have proceeded on the hypothesis that allagochrome is a chlorogenic acid derivative. Briefly, the evidence for involvement of chlorogenic acid in allagochrome synthesis is as follows:

1) It is well known that a green derivative forms spontaneously from chlorogenic acid in the presence of ammonia. The ammonia derivative is spectrophotometrically different from allagochrome. Another chlorogenic acid derivative which forms slowly in the presence of glycine in alkaline medium resembles allagochrome more closely. The latter derivative is reduced to a yellow form by hydrosulfite, is autoxidizable and turns red in acid. In other words it has properties which are descriptively the same as those of allagochrome.

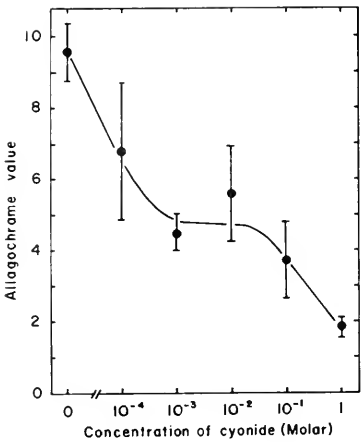
2) The addition of chlorogenic acid during the grinding of plant materials testing positively for allagochrome increases yields up to several fold. Absorption spectra of such enhanced crude preparations match those of control preparations rather well except for slight shifts of peak position in the red and major increases in absorbancy in the blue with a shift to shorter wavelengths for

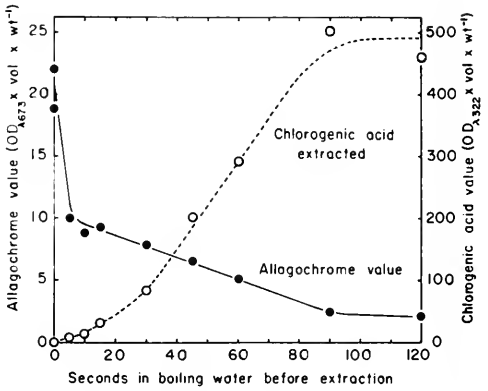
the minimum in difference spectra. Chlorogenic acid extracted from leaves with boiling water is just as effective in increasing yields as the commercially available chemical.

3) The participation of polyphenol oxidase in formation of colored derivatives of chlorogenic acid (especially during aging or after injury of leaves) has been proposed by a number of investigators (8,9). The rapid secondary synthesis of excess allagochrome from chlorogenic acid added during grinding suggested that an enzyme of this type is present and could account for part or all of the pigment found in leaf extracts. The extent of enzymatic formation of allagochrome during grinding was estimated in two ways: by adding cyanide to the extracting medium or by heating leaves in boiling water before extraction. Cyanide added to the extracting buffer reduces allagochrome values (see fig. 1). Heating leaves before extraction similarly reduces allagochrome values in controls and inhibits the secondary synthesis of pigment from added chlorogenic acid. Immersing leaves in boiling water for as short a time as 5 sec. reduces allagochrome values to about half, but longer heating results in proportionately less additional reduction of pigment values of the extracts (see fig. 2). Reduction in allagochrome values is not proportional to removal of chlorogenic acid. Less than 1% is removed by 5 sec. in boiling water and maximum extraction is achieved by this means in 1 1/2 to 2 min. In experiments testing the effects of heating on the secondary synthesis of allagochrome from added chlorogenic acid, only 30 sec. heating before grinding reduced the secondary synthesis to zero; shorter heating resulted in reduction of the secondary synthesis in proportion to the time of treatment over the entire range of added amounts of chlorogenic acid (see fig. 3).

4) Plants testing negatively for allagochrome respond in two possible ways when chlorogenic acid is added during grinding: they either remain negative or become positive. This may be interpreted as an allagochrome negative, enzyme negative vs. allagochrome negative, enzyme positive situation; or it may indicate the presence of a natural antioxidant which interferes with quinone formation. We have not yet resolved this question.

It seems quite probable then that the synthesis of allagochrome from chlorogenic acid and glycine is mediated by an enzyme of the polyphenol oxidase type. This has been substantiated by experiments in which sunflowers were grown on copper deficient nutrient solutions (7). Such plants showed progressively lower copper content of leaves formed at successive nodes and markedly decreased allagochrome levels. At the higher nodes, allagochrome values dropped to between 10 and 20% of controls. There was an increased susceptibility to cyanide poisoning of respiratory oxygen uptake and photosynthetic oxygen production in leaves deficient in copper





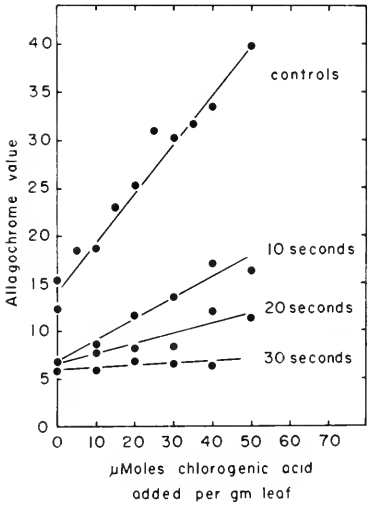


Fig. 1 (upper left). Effects of cyanide in the extracting buffer on allagochrome values of node 3 sunflower leaves. Vertical bars through points indicate standard deviations of means of six determinations..

Fig. 2 (upper right). Effects of immersion in boiling water prior to extraction on allagochrome values of node 8 sunflower leaves. Chlorogenic acid removed was estimated from optical density of aqueous extracts.

Fig. 3 (bottom). Effects of heating on the secondary synthesis of allagochrome from chlorogenic acid added to the extracting buffer. Leaves were immersed in boiling water for 10, 20, or 30 sec. before extraction.

and allagochrome.

Although we now have some working hypotheses concerning the synthesis of allagochrome and evidence concerning its precursor, the reality of the pigment as a component of the living cell remains in doubt. Extraction at high pH, under oxidizing conditions and in a buffer containing glycine provides all the necessary conditions for production of an artifact. Attempts to find another suitable buffer for extraction have been unsuccessful except for borate-NaOH buffer. In this case, allagochrome values of sunflower leaves were only 1/3 to 1/4 what they were with glycine-NaOH buffer. It was not possible to increase yields in borate buffer by adding glycine to the grinding medium. Although there are several indications that at least part of the allagochrome found in extracts of leaves exists *in vivo*, unequivocal proof of its natural occurrence is not yet available.

CHARACTERIZATION OF ALLAGOCHROME

Whether present *in vivo* or not, the pigment allagochrome remains a chemically interesting molecule. The following paragraphs summarize the kinds of information now available on which some speculations concerning the configuration of allagochrome and of the associated yellow pigments can be based. The latter pigments are of increased interest at the present time because of their resemblance to phosphodoxin, a catalyst of photophosphorylation recently reported by Black *et al* [1].

Molecular weight

A preliminary ultracentrifugal analysis was made with a 0.15% solution of allagochrome in a Tris-HCl buffer (pH 8.2). Even prolonged centrifugation at maximum speed (59,780 rpm) did not result in the formation of a boundary and the observed schlieren patterns were characteristic of the transient states observed in an approach to equilibrium. Calculations of the molecular weight were made by a method suggested by Ginsberg *et al* [3] and a value of 720 was obtained. The marked optical density of the solution limited the accuracy attainable by this method. The value should therefore be considered a lower limit and the true molecular weight probably lies between 720 and 1400. This method of analysis gave no information about the homogeneity of the sample but did indicate that no protein was present because of the absence of high molecular weight components.

Electron spin resonance

The first ESR spectra of solid allagochrome and a frozen water solution (containing 2 mg/ml) gave a 12 gaus peak to peak signal at g=2.005. This was the only signal found at that time and on this basis it was concluded that the common paramagnetic metal

ions are not present in purified preparations of the pigment. However, direct chemical analyses of allagochrome had indicated a low but persistent Cu content. These results excluded the possibility that allagochrome might contain Cu^{++}. They also proved that the color changes of allagochrome, especially its reversible reduction, cannot be accounted for on the basis of copper but must rather be accounted for by a probably quinonoid chromophoric group.

ESR signals of the type obtained previously only in the solid have been obtained recently in solution - probably because of increased allagochrome concentration (50 mg/ml vs. 2 mg/ml). This is a free radical signal with some fine structure and present in very low concentrations. The molecule is not all in a free radical form but seems in a state of constant presence of a small amount of free radical. With more concentrated preparations there is a small but recognizable +3 iron signal. No studies have yet been made of the possible effects of light on the ESR signals of allagochrome.

Nuclear magnetic resonance

Table I summarizes information from NMR spectra of a series of pigments and derivatives prepared from Helianthus leaves.

Table I
NMR Spectra of Helianthus Pigment Samples in D_2O

	Sample	Weight(mg)	D_2O(ml)	NMR peaks (in cycles from TMSP)		
1.	Allagochrome	207.5	2	---	214.9	210.5
2.	Allagochrome	54.6	1	---	214.9	209.2
3.	Yellow I	135.3	1.5	---	214.7	---
4.	Yellow I	166.4	1.5	---	214.8	211(broad)
5.	Ppt. from Yellow I in 12N HCl (probably Na acetate)	153.4	2	---	---	---
6.	Supernatant from Yellow I in 12N HCl	224.8	2	233.4	---	---
7.	Yellow II	185.1	2	---	216.2	211.7

It seems probable that all of these compounds except 5 are closely related and may be derivatives of breakdown products of chlorogenic acid plus glycine. These spectra suggest that the quinic acid moiety of chlorogenic acid is not present in the colored products.

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Infrared spectra

Infrared absorption spectra of allagochrome and yellow pigment preparations from Helianthus and Chrysanthemum strongly supported the indications of NMR spectra that these are closely related compounds. Spectra of allagochrome preparations from these two species were nearly identical. A R-CH=CHC(=O)-OR' configuration is definitely not present in allagochrome (no band between 1715 and 1800 cm^{-1}), although such an ester linkage joins together the caffeic and quinic acid portions of the chlorogenic acid molecule. There is no free NH_2 group but there are several indications of bonded N-H of amino or amide, probably of an amide of the type R-C(=O)-N(H)-R'.

Another phenomenon associated with the measurement of infrared spectra is the observation that there is a change in spectrum of allagochrome during the scanning period. Such changes were observed with pigments prepared from both Helianthus and Chrysanthemum leaves. It is not known whether such changes are a consequence of the rather high intensity incandescent illumination received by the samples during measurement of spectra or are merely due to water in the samples.

Elemental analyses

The results of elemental analyses run on a lyophilized sample of Helianthus allagochrome are summarized in Table II.

Table II
Elemental analysis of Helianthus allagochrome

Element	Per cent	
C	24.36%	24.39%
H	4.23%	4.52%
N	12.31%	12.19%
P	0.1 %	
Residue	32.25%	32.19%
Loss at 100°	2.99%	2.38%

The large residue probably indicates a high salt content. We have recently added passage through a mixed bed of ion exchange sephadex as a final step in purification. The resulting samples are much darker green after lyophilization (almost black).

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SUMMARY

The increase in allagochrome values of leaves when chlorogenic acid is added to the extracting medium strongly indicates that this pigment is a chlorogenic acid derivative.

An inhibition of the secondary synthesis of allagochrome from chlorogenic acid by cyanide or heat treatment of leaves is consistent with the hypothesis that this synthesis is mediated by an enzyme of the polyphenol oxidase type. The reduction of allagochrome values in plants deficient in copper adds further support to this hypothesis.

Estimated molecular weight of allagochrome based on analytical ultracentrifuge studies is 720. NMR and infrared spectra indicate that allagochrome and the yellow pigments fractionated from it during purification are closely related chemically. ESR spectra showed a free radical signal at g=2.005.

It remains to be determined whether allagochrome is present *in vivo* or is formed by the enzymatic oxidation of chlorogenic acid during grinding. Present evidence supports the hypothesis that at least part of the assayed amounts of this pigment could be present before extraction.

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SOME EFFECTS OF OXYGEN IN PHOTOSYNTHESIS BY CHLOROPLAST PREPARATIONS

F. R. Whatley

It has been suggested that the presence of oxygen is necessary for photosynthetic phosphorylation. Thus, Nakamoto et al. (1) showed that the cyclic photophosphorylation catalyzed by flavin mononucleotide (FMN) was greatly stimulated by oxygen when very low concentrations of the cofactor were supplied. The formation of ATP in this system was found to be accompanied by an oxygen exchange (2,3), suggesting that, under the experimental conditions employed (compare also ref. 4), the mechanism of the electron transport involved a reaction of reduced FMN with oxygen. But this mechanism cannot be a general one for cyclic photophosphorylation since, as is generally accepted, phenazine methosulfate (PMS) catalyzes a true cyclic photophosphorylation not involving an oxygen exchange under either aerobic or anaerobic conditions (2,3,4). However, even in such a truly cyclic system oxygen has been found to play a role, apparently adjusting the redox balance of the system under some conditions.

Another system in which a part of the photosynthetic apparatus of chloroplasts has been linked with oxygen consumption is the "cytochrome c photooxidase" activity observed by Nieman and Vennesland (5) in digitonin extracts of chloroplasts.

This article discusses two aspects of the participation of oxygen in photosynthetic reactions of chloroplasts: (1) in the photooxidation of ferrocytochrome c by digitonin extracts of chloroplasts and (2) as a "poising agent" to regulate electron flow in the true cyclic photophosphorylation catalyzed by PMS in broken chloroplasts.

(1) Photooxidation of reduced cytochrome c.

Confirming the results reported by Nieman and Vennesland (5), it was found that digitonin extracts of chloroplasts (prepared by continued extraction of spinach chloroplasts by 1% digitonin, followed by the centrifugation procedure described by Nieman

and Vennesland) oxidized ferrocytochrome c at a slow rate in the dark in air but not in argon, and that this dark oxidation, which proceeded in accordance with the thermochemical gradient, was completely suppressed by 10^{-4} M KCN. Ferrocytochrome c was, however, oxidized when the system was illuminated in the presence of KCN-treated digitonin extracts and oxygen was supplied. No photooxidation occurred under an atmosphere of argon or in the dark, as shown in Fig. 1. Nieman and Vennesland concluded from similar evidence that a "cytochrome c photooxidase" had been unmasked in their preparation. However, as will now be reported, triphosphopyridine nucleotide (TPN) can be substituted for oxygen as a terminal electron acceptor in an atmosphere of argon. To accomplish this it was necessary to add both the electron carrier ferredoxin and the enzyme ferredoxin-TPN reductase to transfer the electrons produced by the photoreaction to TPN. An experiment showing the photooxidation of ferrocytochrome c by TPN under argon is shown in Fig. 2 and represents another experimental manifestation of Nieman and Vennesland's apparent cytochrome c photooxidase activity.

Photooxidation of ferrocytochrome c by TPN proceeded against a thermochemical gradient, unlike the reaction with oxygen, and obviously required an input of light energy. It was not immediately apparent why the oxidation of ferrocytochrome c by oxygen should also need an input of light. However, our results suggest that the photooxidation of ferrocytochrome c is a manifestation of the terminal portion of the electron transport chain of noncyclic photophosphorylation in chloroplasts (see ref. 6), in which TPN acts as the physiological electron acceptor, but can be replaced unspecifically by molecular oxygen. Additional support for the view that the digitonin extracts retained the terminal portion of the electron transport chain comes from an experiment, shown in Fig. 3, in which ferrocytochrome c was replaced by the ascorbate/dichlorophenol indophenol dye couple. In the light, but not in the dark, TPN became photoreduced at the expense of the oxidation of ascorbate. A preliminary report of these results has appeared elsewhere (7).

(2) Cyclic photophosphorylation catalyzed by phenazine methosulfate.

It is generally accepted that PMS catalyzes a truly cyclic electron flow in chloroplasts, accompanied by ATP formation (2-4). The principal evidence to support this conclusion may be summarized: (i) No oxygen exchange was observed to accompany the ATP formation (2,3), (ii) the rates of phosphorylation in

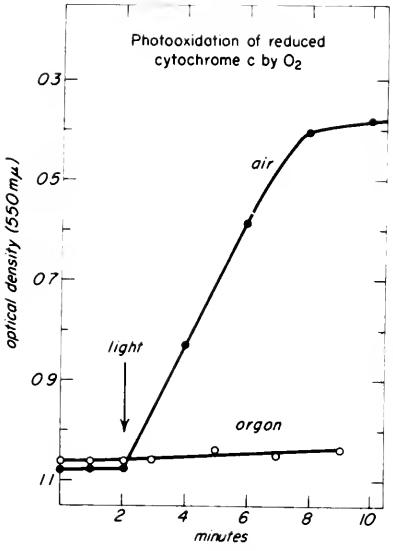


Fig. 1. Experimental details as described by Nieman and Vennesland (5). A decrease in O.D. at 550 μm corresponds to the oxidation of ferrocytochrome c.

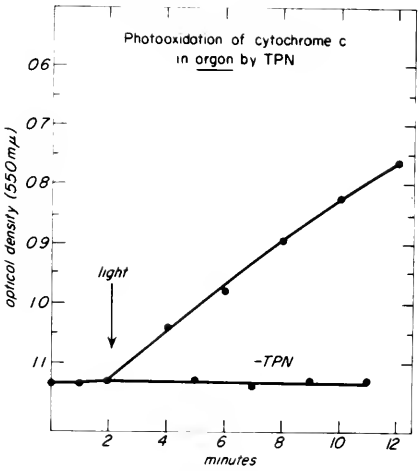
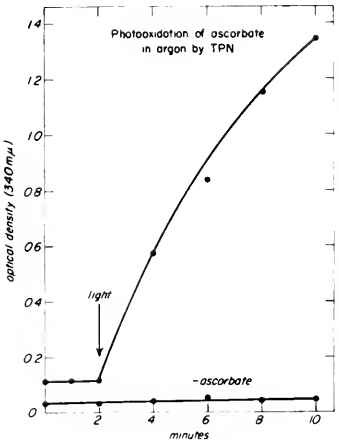


Fig. 2. Experimental details as in Fig. 1, except that a partially purified preparation of "photosynthetic pyridine nucleotide reductase" (11) was added to each vessel, and 1 μmole TPN was added as indicated.

Fig. 3. Experimental details as in Fig. 2, except that ferrocytochrome c was replaced by 20 μmoles ascorbate + 0.2 μmoles dichlorophenol indophenol (both omitted in "-ascorbate" treatment). Increase in O.D. at 340 mμ corresponds to reduction of TPN.



air and under nitrogen were similar at saturating concentrations of PMS, and (iii) there was little effect of CMU on the rate of ATP formation anaerobically with saturating PMS (4). However, some unexpected effects of oxygen on the PMS-catalyzed cyclic photophosphorylation have been found. These effects will be discussed in the context of an interpretation proposed by Tagawa et al. (8) of the effect of oxygen on the cyclic photophosphorylation catalyzed by ferredoxin.

In Table 1 is shown the effect of increasing the concentration of the cofactor, PMS, on the formation of ATP in argon or in air. Although at the saturating concentration of PMS commonly employed (30 µgrams per 3 ml reaction mixture) there was no difference between the rate in air or argon, a large stimulatory effect of air became apparent at lower concentrations of PMS. When 3 µgrams PMS were used the reaction was not saturated by PMS (under our experimental conditions), but there was a threefold stimulation by air of the ATP formation. A similar stimulation has also been observed by Jagendorf and Avron (9). The addition of small amounts of oxygen was found to produce the same effect as air. In Table 2 are reported the results obtained when 3 µgrams PMS were used as the cofactor for cyclic photophosphorylation and small amounts of oxygen gas were added to a reaction vessel of approximately 20 ml capacity. When 10 µmoles oxygen had been added the rate of ATP formation was max-

Table 1

Effect of air on cyclic photophosphorylation catalyzed by PMS

PMS added (µgrams)	ATP formed in 15 min. (µmoles) Argon	Air
0	0.3	0.4
0.3	0.2	0.6
1	0.4	1.2
3	1.1	3.8
10	7.6	8.8
30	8.7	9.2

The reaction mixture contained, in a final volume of 3 ml, broken chloroplasts (P_{1S}) containing 0.1 mg chlorophyll and the following in micromoles: tris buffer, pH 8.3, 80; $MgCl_2$, 10; ADP, 10; $K_2HP^{32}O_4$, 10; and the amounts of phenazine methosulfate indicated. The experiments were carried out in Warburg manometer vessels at 15° C at 20,000 lux. Prior to turning on the light, the "argon" series was flushed with purified argon gas for 5 minutes. ATP formation was measured as described previously (11).

imal, but smaller amounts of oxygen produced a large stimulation. 10 μmoles oxygen added to a vessel of 20 ml volume corresponds to about 1% oxygen in the gas phase.

Table 2

Effect of small amounts of oxygen on cyclic photophosphorylation catalyzed by a limiting amount of PMS under argon

Oxygen added (μmoles)	ATP formed in 30 minutes (μmoles)
0	1.7
2	4.7
4	5.6
10	7.8

Experimental conditions were as given in legend to Table 1. 3 μgrams PMS were added in each vessel. The reaction vessels were capped with a serum bottle cap and flushed with purified argon. Small amounts of oxygen gas were then injected into the gas phase through the cap.

These results appear similar to those obtained with the FMN-catalyzed pseudo-cyclic photophosphorylation, which has been shown to require oxygen as a terminal electron acceptor. As already stated, however, the oxygen exchange data do not support a similar interpretation for the PMS system. Consequently, an alternative explanation was sought. If the effect of oxygen in the PMS system were due to the oxidation of some component of the system, it seemed probable that the addition of another oxidant would produce a similar stimulation of the ATP formation. Since ferricyanide is not toxic to photophosphorylation by chloroplasts (e.g., it supports ATP formation in noncyclic photophosphorylation) it was chosen as a suitable substitute for oxygen. When tested in a system provided with a limiting amount (3 μgrams) of PMS, ferricyanide was indeed capable of stimulating cyclic photophosphorylation with PMS under argon, as is shown in Table 3. The table indicates very clearly that the ferricyanide acts in a "catalytic" fashion, and not as a substrate; the addition of 1 μmole ferricyanide (which in noncyclic photophosphorylation might have given 0.5 μmoles ATP) caused an increment of 5 μmoles ATP. The effect of ferricyanide is not due to the oxygen which it will produce via a Hill reaction when the light is turned on. This will be seen by comparing the results in Tables 2 and 3 (both experiments carried out at the same time); 1 μmole ferricyanide, which gave a large stimulation, would yield 0.25 μmoles oxygen, an amount much less than is needed to give a measurable effect when injected into the gas phase.

Table 3

Effect of small amounts of ferricyanide on cyclic photophosphorylation catalyzed by a limiting amount of PMS under argon

Ferricyanide added (μmoles)	ATP formed in 30 minutes (μmoles)
0	1.7
0.25	3.5
0.5	6.2
1.0	6.7
1.5	7.9
2.0	8.0
2.5	8.4

Experimental conditions as described in legend to Table 1. 3 μg PMS were added to each vessel. Ferricyanide was added as indicated and remained in contact with the chloroplast fragments and the PMS for 5 minutes while the vessels were flushed with argon, and before the light was turned on.

The inhibitor p-chlorophenyl-dimethyl-urea (CMU) also affected cyclic photophosphorylation with PMS, as can be seen by examining the data presented in Table 4. The addition of CMU (2.10^{-5} M final concentration) to the PMS system under argon

Table 4

Effect of CMU on cyclic photophosphorylation catalyzed by PMS in air or argon

PMS added (μgrams)	ATP formed in 15 min (μmoles) 1 argon	2 argon + CMU	3 air	4 air + CMU
0	0.3	0.3	0.4	0.2
0.3	0.2	0.6	0.6	0.2
1	0.4	1.7	1.2	0.6
3	1.1	5.8	3.7	1.1
10	7.6	8.9	8.8	1.9
30	8.7	9.2	9.2	3.7

Experimental conditions as described in legend to Table 1. CMU to a final concentration of 2.10^{-5} M was added where indicated.

produced a large stimulation of ATP formation at lower concentrations of PMS (Columns 1,2), this effect resembling very closely the effect of air (Column 3). When 30 μgrams PMS were added no effect was produced by CMU. On the other hand, the

addition of CMU to the PMS system in air caused a profound inhibition of ATP formation (Column 4), which was only partly overcome by 30 μgrams of PMS. Jagendorf and Avron (9) have made similar observations with both CMU and o-phenanthroline. These results do not at first appear to support the conclusion that PMS catalyzes a truly cyclic photophosphorylation, but are compatible with this conclusion when the data are interpreted in terms of a "poising" action of oxygen (redox regulation).

In a recent paper Tagawa et al. (8) have advanced such an hypothesis to explain their results on cyclic photophosphorylation catalyzed by ferredoxin. They point out that noncyclic electron flow is a unidirectional electron transfer from water to TPN, driven by two photoreactions, B and A (systems 2 and 1, respectively, in Duysens' terminology (10)). The intermediates in the electron transport chain will be kept in a partly reduced, partly oxidized state (i.e., "poised") as long as TPN (or ultimately CO_2) is available, and no "overreduction" or "overoxidation" can occur. However, to maintain a cyclic electron flow from a reduced cofactor back to the electron transport chain, as is required for cyclic photophosphorylation, the intermediates of the electron chain must be kept in a suitable redox balance. If they are kept fully reduced, as by photoreaction B, they cannot accept electrons from the reduced cofactors. Tagawa et al. proposed the hypothesis "that molecular oxygen normally regulates the redox balance for the electron transport chain involved in cyclic photophosphorylation by chloroplasts. In the presence of oxygen, the electron transport system does not become overreduced by the flow of electrons from water. But under anaerobic conditions the flow of electrons from photoreaction B overreduces the components of the electron transport chain in chloroplasts and this overreduction cannot be counterbalanced by the regulatory oxidizing action of oxygen."

As shown in Table 1, the rate of ATP formation at lower concentrations of PMS was much less under argon than in air. This is interpreted to mean that, under argon, electrons flowing from photoreaction B overreduce the intermediates of the electron transport chain; and this slows down or prevents the return of electrons from photoreaction A via PMS to the chain. Oxygen can counterbalance the overreduction by oxidizing some portion of the intermediates and so bring about a regulation of the electron flow. Smaller amounts of oxygen produce a proportionately smaller response, although the action of oxygen is clearly catalytic (Table 2). The fact that very small amounts of ferricyanide (Table 3) stimulate the cyclic photophosphorylation with

PMS in a similar fashion emphasizes the idea that redox reagents other than oxygen can regulate ("poise") the system by partly oxidizing the intermediates. It appears likely from the results in Table 1 that even PMS itself in larger amounts (10-30 μgrams) can bring about the redox regulation of the electron transport chain. When photoreaction B was prevented by the addition of the specific inhibitor, CMU, the flow of electrons from water to the electron transport chain was stopped, and no overreduction could occur. There was thus no need for oxygen as a regulator under argon in the presence of CMU (Table 4). On the other hand, in the presence of air some contribution of electrons from water by photoreaction B appears to be necessary in order to maintain a suitable redox balance. The addition of CMU caused a large inhibition of cyclic photophosphorylation in air (Table 4), probably because oxygen overoxidized the intermediates of the electron transport chain, thus preventing a supply of electrons to photoreaction A.

A possible explanation of the result shown in Table 1 might have been that in air the PMS is converted to the oxidation product pyocyanine (cf. Hill and Walker, 13) and that pyocyanine functions more effectively as a catalyst at lower concentrations than PMS. However, as is shown in Table 5, pyocyanine-catalyzed cyclic photophosphorylation behaves like the PMS catalyzed system in its reaction toward aerobic and anaerobic conditions and towards CMU.

Table 5

Effect of CMU and of air on cyclic photophosphorylation catalyzed by limiting amounts of pyocyanine

Pyocyanine added (micrograms)	ATP formed in 15 minutes (μmoles) air	argon	argon + CMU	air + CMU
1	1.2	0.5	1.1	0.4
2	2.9	0.7	2.1	0.5
3	4.3	0.7	3.6	0.4

Experimental conditions as described in legend to Table 4, except that pyocyanine was substituted for PMS.

Thus with PMS or pyocyanine as the cofactor, just as with ferredoxin, oxygen is able to play a regulatory role to combat the apparent tendency of photoreaction B to overreduce the intermediates of the electron transport chain. When photoreaction B is suppressed the system is already in a suitable

redox balance without oxygen, and in the case of both PMS and pyocyanine can be shown to become easily overoxidized by oxygen. The hypothesis of a regulatory role for oxygen is consistent with the results of Jagendorf and Avron on the effect of air, CMU and o-phenanthroline on the PMS system, for which they were earlier unable to offer an explanation (9). The hypothesis also suggests an explanation for the stimulatory effect of DCMU on the cyclic photophosphorylation catalyzed by the indophenol dyes, which has been described by Trebst and Eck (12).

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III. STUDIES WITH ISOLATED ELECTRON CARRIERS

PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE REDUCTASE. IV FURTHER STUDIES ON THE CHEMICAL PROPERTIES OF THE PROTEIN

Keelin T. Fry and Anthony San Pietro

The first demonstration that a soluble factor can be added back to chloroplasts to reconstitute their over-all electron-transport reaction was reported by Davenport, Hill and Whatley [1] in 1952. They showed that washed chloroplasts lost their capacity for reducing muscle methaemoglobin in the light. The addition of the soluble fraction restored the activity. They further demonstrated that the soluble methaemoglobin reducing factor was thermolabile.

Lang and San Pietro [2, 3] were unaware of the prior work of Davenport et al. [1] when they reported that chloroplasts contain a soluble protein which catalyzed the photochemical reduction of pyridine nucleotides. At that time there was no evidence to suggest that the proteins isolated independently by these two groups might be identical. The possible identity of these two proteins, namely, photosynthetic pyridine nucleotide reductase (PPNR) and the methaemoglobin reducing factor, only became apparent when they were available in purified form [4, 5].

During the course of their studies on the methaemoglobin reducing factor, Davenport and Hill [6] observed that the preparation catalyzed the reduction of a number of haem-proteins, including cytochrome c, by illuminated chloroplasts. Moreover, this factor was shown to catalyze the reduction of NADP by illuminated chloroplasts [7].

At the suggestion of Dr. H. E. Davenport, the ability of PPNR to catalyze the reduction of cytochrome c by illuminated chloroplasts was examined and it was found to catalyze this reduction [4]. Thus, the catalytic activities of these two proteins are identical.

ABSORPTION SPECTRUM

The methaemoglobin reducing factor [6] and PPNR [8] have been extensively purified and shown to be homogeneous, both electrophoretically and in the ultracentrifuge. Both proteins contain two moles of non-haem iron per mole of enzyme and an equivalent amount of "labile sulfur" [9-11]. The absorption spectra of PPNR from spinach and of the methaemoglobin reducing factor from parsley are shown in Figure 1. It is clear that these two spectra are very similar; the major difference between them is the relationship of the absorption in the visible region to that in the ultra-violet region. The

ratios of absorbance in the visible region to that in the ultra-violet region (277 mμ) for the absorption maxima at 330 mμ, 420 mμ and 465 mμ are 0. 81, 0. 62 and 0. 57 for the methaemoglobin reducing factor and 0. 65, 0. 49 and 0. 44 for PPNR. In each case, the values for the methaemoglobin reducing factor are about 1. 27 times the corresponding value for PPNR. This difference is most probably due to the fact that the methaemoglobin reducing factor is devoid of tryptophane [11] whereas PPNR contains one mole of tryptophane (Table 2). The presence of tryptophane in PPNR is indicated by the shoulder in the absorption spectrum at 290 mμ which is absent from the absorption spectrum of the methaemoglobin reducing factor. In addition, there is a shift in the position of the minimum at 295 mμ in the spectrum of the methaemoglobin reducing factor to 305 mμ in the PPNR spectrum.

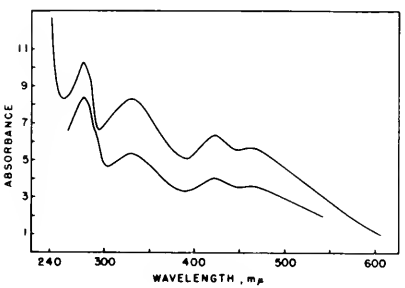

Fig. 1. Absorption spectra of methaemoglobin reducing factor from parsley and PPNR from spinach. Upper curve, methaemoglobin reducing factor, 0. 77 mg of protein per ml; Lower curve, PPNR equivalent to 0. 081 micromole of iron per ml.

Recently, Katoh and Takamiya [12] reported the isolation of PPNR from Brassica campestris (Komatsuna). The absorption spectrum of the Brassica PPNR corresponds almost identically to that of the methaemoglobin reducing factor from parsley. The relative absorption values at 276, 330, 420 and 465 mμ are 1. 00, 0. 81, 0. 60 and 0. 54 for the PPNR from Brassica. These correspond very well with those given above for the methaemoglobin reducing factor. We would suggest, therefore, that the PPNR from Brassica will also be found to be devoid of tryptophane.

In view of the difference in tryptophane content of these various proteins, any comparison of their ultra-violet absorption spectra per unit of protein will be meaningless. However, the absorption in the visible region per micromole of iron should be comparable provided the iron in these proteins is an essential constituent of the chromophoric group(s) and that the chromophoric group(s) in these proteins are the same. The absorbance per micromole of iron per milliliter at the wavelengths of the absorption maxima in the visible region is presented in Table 1. For the purposes of comparison,

similar data for the spinach ferredoxin isolated in Arnon's laboratory [13, 14], the red enzyme from Chlorella isolated by Gewitz and Volker [15] and the PPNR isolated from Japanese spinach by Horio and Yamashita [16] are included. Except for the PPNR from Brassica and Japanese spinach, the values at each wavelength for the various proteins are almost identical. These data provide support for the hypothesis that the chromophoric group(s) responsible for the visible absorption of these proteins is the same.

It is interesting that the extinction coefficients per iron at 390 mμ for the Clostridial ferredoxins are approximately the same as those reported in Table 1 for the absorption at 465 mμ. From the data of Buchanan et al. [17] one can calculate that the extinction coefficients of the Clostridial ferredoxins at 390 mμ per micromole of iron per milliliter are about 3.5-4.5 assuming a value of 12,000 for the molecular weight and an iron content of 10 atoms of iron per mole.

TABLE 1

Correlation of Absorbance in the Visible Region and Iron Content

The values are presented as the absorbance of a solution of protein containing one micromole of iron per milliliter.

Protein	Source	Wavelength (mμ) 330	420	465
PPNR[a]	Spinach	6.70	4.95	4.45
Red Enzyme[b]	Chlorella	7.37	5.29	4.89
Spinach Ferredoxin[c]	Spinach	6.95	5.16	4.65
Methaemoglobin Reducing Factor[d]	Parsley	6.83	5.18	4.67
PPNR[e]	Brassica	3.43	2.57	2.32
PPNR[f]	Spinach	11.7	8.94	8.60

[a]Calculated from the lower curve in Fig. 1.
[b]Calculated from the data of Gewitz and Volker [15].
[c]Calculated from the data of Whatley, Tagawa and Arnon [13] and Tagawa and Arnon [14].
[d]Calculated from the data of Bendall, Gregory and Hill [9] and the upper curve in Fig. 1.
[e]Calculated from the data of Katoh and Takamiya [12].
[f]Calculated from the data of Horio and Yamashita [16] assuming a molecular weight of 14,000.

AMINO ACID COMPOSITION

The amino acid composition of PPNR from spinach is given in Table 2. The protein is acidic as evidenced by the high concentration of aspartic and glutamic acids and the low concentration of the basic amino acids.

TABLE 2

Amino Acid Analysis of Spinach PPNR

Amino Acid	Residues per Mole
Lysine	4
Histidine	1
Arginine	1
Aspartic Acid	12
Threonine	8
Serine	6-7
Glutamic Acid	13-15
Proline	4
Glycine	7
Alanine	9
Valine	7-8
Isoleucine	4
Leucine	7
Tyrosine	4
Phenylalanine	2
Tryptophane	1
Methionine	1
Ammonia	(8-10)

For the determination of tryptophane, samples of protein were hydrolyzed with 5 N NaOH containing basic lead acetate [18] by heating for 18 hours at 100° in evacuated glass ampules. Tryptophane content of the hydrolysates was determined by the method of Spies and Chambers (Procedure Q) [18]

Preliminary analyses of the cysteine content of the protein indicate the presence of about six half-cystine residues determined as cystein acid following oxidation with performic acid [19].

REACTION WITH p-CHLOROMERCURIBENZOATE (PCMB)

Treatment of PPNR with PCMB results in a rapid loss in the visible spectrum of the enzyme. As shown in Figure 2, there is a parallel loss in absorbance at each of the three absorption maxima in the visible region. After complete reaction, the percentage of the original absorbance remaining at 330, 420 and 465 mμ was 40%, 10% and 5%, respectively.

The decrease in color with addition of mercurial can most easily be interpreted as resulting from the disruption of the iron-protein chromophore. The amount of PCMB required for complete titration (Figure 2) was 0.47 μmole. This amount of PCMB corresponds to about 8-9 equivalents of PCMB reactive groups per mole of enzyme. The same result was obtained by a sulfhydryl titration of PPNR according to the method of Boyer [21]. These values are higher than expected on the basis of the preliminary estimate for

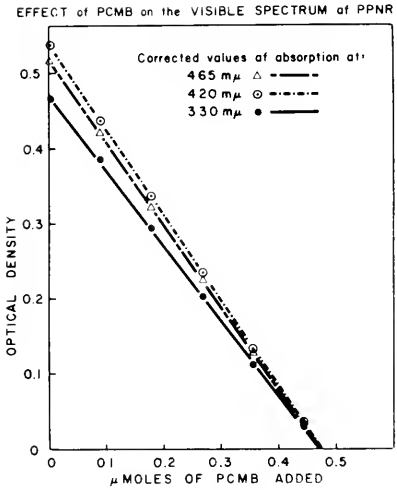

Fig. 2. Effect of PCMB on the visible spectrum of PPNR. The reaction mixture contained 1.13 mg of PPNR, determined by the method of Warburg and Christian [20], in 0.9 ml of 0.05 M Tris, pH 7.45. The values were corrected for absorption remaining after complete titration. PCMB concentration was determined spectrophotometrically at 232 mμ as described by Boyer[21].

the half-cystine content of PPNR from cysteic acid analyses and may reflect a reaction between PCMB and the "labile sulfur" shown previously to be present in the enzyme.

Similar titrations of PPNR with mercurials have been performed by other investigators [12, 22]. Katoh and Takamiya [12] interpret their results to indicate the presence in PPNR from Brassica leaves of 12 sulfhydryl groups. This interpretation assumes no reaction between mercurial and "labile sulfur".

TREATMENT WITH o-PHENANTHROLINE (OP) [10]

In addition to iron, PPNR contains "labile sulfur" which is liberated as hydrogen sulfide upon acidification. Assays for the sulfide were performed by applying the method of Fogo and Popowsky [23] directly to solutions of the protein. The molar ratio of iron to labile sulfur with several preparations averaged from 0.9-1.1. Control experiments indicate that cysteine groups in the protein are not the source of the labile sulfur.

To determine the changes resulting from treatment with the iron chelator OP, the enzyme was incubated with the chelator and then, at varying time intervals, separated from the latter, as well as from the ferrous triphenanthrolate complex which formed, by chromatography on Sephadex G-25. Analyses of protein fractions obtained in such an experiment are summarized in Table 3. The data indicate that under conditions of pH and temperature at which PPNR is relatively stable, treatment with OP results in loss of iron from the enzyme as well as a corresponding loss of "labile sulfur", absorption in the visible region of the spectrum and activity of the enzyme in TPN reduction.

TABLE 3

Properties of PPNR following incubation at 25° and pH 8 in the presence or absence of o-phenanthroline.

Sample -OP	Sample +OP	Reaction Time in Minutes	Percentage Remaining					
			Iron	Labile Sulfur	Activity in TPN Reduction	Absorbance at: 329 mμ	420 mμ	465 mμ
1		7	93	92	93	99	100	101
2		264	92	80	85	92	90	90
	3	7	89	84	90	94	94	95
	4	46	65	67	66	75	73	73
	5	92	51	51	55	61	58	57
	6	142	37	37	34	49	42	41
	7	248	17	15	12	32	22	21
	8	ca. 1400	4	--	--	17	5	4

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The correlation between the percentage of iron lost and the loss of absorption in the visible region of the spectrum is actually better than is apparent from the values reported in Table 3 since there is apparently some residual absorption in this region even in iron-free enzyme.

OXIDATION STATE OF IRON

Although incubation of PPNR with OP results in removal of iron from the protein and formation of the ferrous triphenanthrolate complex, this observation does not constitute proof that the metal in the native enzyme is in the ferrous state since reduction of bound ferric iron could occur before, during, or after release from the protein. Removal of the metal from the native enzyme by the ferric iron chelator Tiron (1, 2-dihydroxybenzene-3, 5-disulfonate) is negligible under comparable conditions. If, however, the PPNR is first treated with the sulfhydryl reagent p-chloromercuriphenyl sulfonic acid (PCMS), there is a rapid reaction with the ferric chelator as shown by the results of an experiment reported in Table 4. In this

TABLE 4

Reaction of PPNR with OP or Tiron following addition of PCMS

Time in minutes after addition of chelator	% Reaction with OP	% Reaction with Tiron
2	6	96
10	8	102
30	12	104
120	28	
900		110

Reaction mixture contained PPNR equivalent to 0. 35 μmoles of iron, 52 μmoles of Tris· HCl buffer, pH 8, 2. 9 mg of glucose oxidase (Sigma, type II), 29 mg of glucose and 0. 3 mg of crystalline horse liver catalase in a total volume of 4. 5 ml. Additions were 6 μmoles of PCMS in 0. 3 ml of 0. 2 M Tris· HCl buffer, pH 8 and either 4. 5 μmoles of OP in 0. 3 ml of 0. 1 M Tris· HCl buffer, pH 8, or 4. 5 μmoles of Tiron (adjusted to pH 8) in 0. 3 ml of 0. 1 M Tris· HCl buffer, pH 8.

experiment the possibility of air oxidation of any ferrous iron present in the protein was minimized by carrying out the reaction with the chelators under anaerobic conditions in glass cuvettes fitted with a ground glass capillary stopper. PPNR and an oxygen trapping system of glucose-glucose oxidase were preincubated in the completely filled cuvettes for 20 min. at 25° before addition of, in order, PCMS and either Tiron or OP. Before use, the PCMS and chelator solutions were freed from oxygen by bubbling with argon for 15 min. The mercurial and the chelators were added to the cuvettes from a syringe through the capillary bore of the glass stopper. Thus an amount of

solution equivalent to the volume of the added reactant was forced from the cuvette at each addition. Formation of the ferrous triphenanthrolate complex was determined from the absorbance at 510 mμ (E = 11, 100) [24] and formation of the ferric complex with Tiron from absorbance at 487 mμ (E = 5, 800 as determined at pH 8). The latter extinction coefficient corresponds well with the value of 6, 100 calculated from the data of Yoe and Jones [25] and was used, therefore, to calculate the values presented.

Control experiments in which ferrous iron was added to the anaerobic reaction mixtures after treatment with PCMS indicated that the ferric iron observed did not arise from oxidation of free ferrous iron in solution. Thus, it would appear from the data in Table 4 that the metal in the native enzyme is in the ferric oxidation state. The color formation observed with OP probably results from reduction by reducing agents present in the protein which are not bound by the mercurial. In this regard it should be noted that the amount of PCMS added was about four times that required to titrate the PPNR.

PHOTOREDUCTION OF IRON [26]

It has been demonstrated by several investigators that PPNR can be photoreduced in the presence of illuminated chloroplasts [12, 13, 16, 27]. It was of interest, therefore, to determine whether reduction of the enzyme was accompanied by a concomitant change in the valence state of the non-haem iron. For this purpose reaction mixtures containing PPNR and chloroplasts were illuminated anaerobically for varying lengths of time. After illumination, the extent of photoreduction and the appearance of ferrous iron were determined [26]. As shown in Table 5, one-half the iron is in the ferrous state when the protein is completely reduced.

TABLE 5

Photoreduction of Iron

Illumination Time in Minutes	Per Cent Bleaching 420 mμ	460 mμ	Per Cent of Total Iron in Ferrous State	% Fe^{++}/Average % Bleaching
0.5	34	32	19	0.58
1.0	74	74	33	0.45
3.0	97	97	44	0.45

PHOTOREDUCTION BY CHLOROPLASTS

Chance and San Pietro [27] investigated the photoreduction of PPNR by chloroplasts and showed that the light-dark difference spectrum for PPNR reduction by illuminated chloroplasts corresponded to the difference spectrum for dithionite-reduced PPNR. The kinetics of reduction of PPNR by

illuminated chloroplasts were measured and it was found that PPNR was reduced more rapidly than NADP. The kinetics of reduction of PPNR by illuminated chloroplasts indicated that the reduced form has the potentiality of acting as an electron carrier between chloroplasts and NADP.

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PHOTOCHEMICAL REACTIONS OF PLASTOCYANIN IN CHLOROPLASTS

Sakae Katoh and Atusi Takamiya

The idea that copper functions in photosynthesis in green plants was proposed before it was shown to be present in the photosynthetic apparatus. In 1939, Neish first described the occurrence of copper in chloroplasts in a remarkably high concentration when compared with the other metals present[1]. In the same year, Green et al. showed that the photosynthetic carbon dioxide uptake was markedly inhibited by several reagents which were generally regarded as inhibitors of copper enzyme[2]. The relatively high effectiveness of these inhibitors, at concentrations which were completely without effect on the dark respiration, led these authors to propose that copper is involved in the photosynthetic reactions of chlorella.

Since then, several reports appeared in confirmation of these earlier results. Whatley et al. showed a high localization of copper in the chloroplast fragments of sugar beet[3]. The sensitivity of photosynthesis and the Hill reaction towards the copper-binding reagents has also been repeatedly confirmed with chloroplasts[4, 5]. Furthermore, Spencer and Possingham, working with tomato plants, clearly demonstrated that copper is an essential micronutrient for the development of chloroplast activity[6].

Prior to the work reported here it was thought that the copper in the chloroplasts was associated with those proteins which oxidize polyphenols[7]. To date, however, there is no conclusive evidence for the participation of this group of enzyme in the photochemical processes of chloroplasts. In addition they are not ubiquitous in occurrence among photosynthetic plants and algae. Moreover, when they are present, their concentration in the chloroplasts is generally low and varies widely with different plants.

Another type of copper complex, which we have recently discovered in the chloroplasts, is a non-autooxidizable copper protein, plastocyanin[8]. It is proposed that this copper protein functions in the photochemical activity of the chloroplasts. The aim of the present work is to summarize the general properties of plastocyanin, as well as its distribution in photosynthetic organisms. The results of our recent experiments on the effect of this copper protein on several photochemical reactions of chloroplasts will be discussed.

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GENERAL PROPERTIES OF PLASTOCYANIN

Plastocyanin is readily extracted with dilute buffer solution from an acetone powder of a leaf homogenate. It is purified by ammonium sulfate fractionation and column chromatography with diethylaminoethyl cellulose[9]. Crystallization of the protein from Chenopodium leaves has been achieved by Yakushiji (personal communication). The molecular weight of the purified protein was estimated from sedimentation and diffusion data to be 21, 000[9]. The protein is acidic in nature with an isoelectric point less than pH 4.

The oxidized protein is deep blue in color and exhibits a complex absorption spectrum with three maxima at 460, 597 and 770 mμ. The ultraviolet peak shows vibrational fine structure bands due to the amino acid constituents. On reduction, the blue color completely disappears and there is no absorption in the visible and far red regions. The oxidized protein is readily reduced by various reducing reagents such as ascorbaic acid, hydroquinone, sodium hydrosulfite and reduced cytochrome c. On the other hand, the reduced form of the protein is completely incapable of reacting with molecular oxygen through oxidation or oxygenation. The oxidation reduction potential is 370 mv between pH 5.4 and 9.9.

Plastocyanin contains about 200 amino acid residues and a small amount of carbohydrate. The copper content was estimated to be 0.58%, indicating the presence of two atoms of copper. Titration with either PCMB or heavy metal ions indicated that each copper atom is bound to the protein through the sulfhydryl groups of cysteine. The original copper protein was reconstituted by addition of an inorganic copper salt to the apoprotein prepared by acid treatment.

DISTRIBUTION OF PLASTOCYANIN IN PLANTS

Plastocyanin was first observed in the aqueous extract of lyophylized Chlorella elipsoidea[8]. The green leaves of the following plants were found to contain plastocyanin in significant amounts; spinach, parsley, carrot, turnip, crown daisy, Japanese scallion, Brassica campestris (Komatsuna), Chenopodium album and Ulva sp.[10]. Plastocyanin appears to occur ubiquitously in the photosynthetic organisms. One exception was the photosynthetic bacteria, since all attempts to detect a similar protein has failed.

The occurrence of plastocyanin is limited to the green parts of the plants. The protein is entirely absent from the white underground stem of Japanese scallion and the roots of carrot and turnip, while the green leaves of these plants contain significant amounts of the copper protein. This fact suggests a close association of plastocyanin with the photosynthetic apparatus. In fact, whole chloroplasts isolated from spinach leaves were found to contain plastocyanin in a ratio of about 300 chlorophyll molecules per atom of copper of the protein, or about 600 chlorophyll molecules per molecule of the protein. Since the total copper content of spinach chloroplast was estimated to be approximately

130 - 160 chlorophyll molecules per atom of copper, the copper in plastocyanin can account for about half of the total copper in the chloroplasts. It is noteworthy that the concentration of plastocyanin expressed on a chlorophyll basis is similar in magnitude to that of cytochrome f[11].

It was found that hypotonic treatment of whole chloroplasts resulted in the release of a significant portion of plastocyanin. The grana fraction also contained plastocyanin at a concentration comparable to that in the whole chloroplast. The grana also lost some plastocyanin by hypotonic treatment, thus indicating the association of the protein with the subparticulate fraction of the chloroplasts. All these findings strongly suggest that the protein under investigation is an integral part of the photosynthetic architecture of chloroplasts.

PHOTOREDUCTION OF PLASTOCYANIN BY CHLOROPLASTS

When oxidized plastocyanin was incubated with chloroplasts in the light, a steady decrease in absorbance at 597 mμ was observed[12]. Upon addition of ferricyanide at the end of the reaction, the blue color was restored to the original level, thereby indicating that the decrease in absorbance was correlated with reduction of the protein. Neither reduction nor oxidation of plastocyanin was observed during incubation with chloroplasts in the dark.

The rate of photoreduction usually obtained ranged from 100 to 200 μmoles protein per mg chlorophyll per hour[13]. The rate increased with light intensity and reached saturation at about 10,000 lux. The reaction was optimal between pH 7.5 - 7.9. The reaction was completely inhibited by 10^{-6} M DCMU. o-Phenanthroline and hydroxylamine were also potent inhibitors of the photoreduction. Cyanide, azide and arsenite were without effect at a concentration of 10^{-3} M. The rate of photoreduction was increased by the presence of $MgCl_2$ and ADP (the reaction system contains a sufficient amount of phosphate), indicating that the reaction is coupled with phosphorylation. A very marked stimulation of rate of reduction was observed on addition of ammonium sulfate, which is known to uncouple the photophosphorylation from the electron transferring system of the chloroplasts[14]. It may be concluded from these observations that plastocyanin is reduced by illuminated chloroplasts by the same pathway as that of photoreduction of ferricyanide and NADP.

Photooxidation of Reduced Plastocyanin by Digitonin-Treated Chloroplasts

It was found that reduced plastocyanin was readily photooxidized by chloroplasts which had been treated with digitonin. A similar chloroplast preparation was shown by Nieman and Vennesland to catalyze the photooxidation of reduced cytochrome c[15]. The time course of the photooxidation of reduced plastocyanin by digitonin-treated chloroplasts measured by the increase in absorbance at 597 mμ is illustrated in Fig. 1. No change in oxidation reduction state of the added plastocyanin was observed in the dark. The removal of

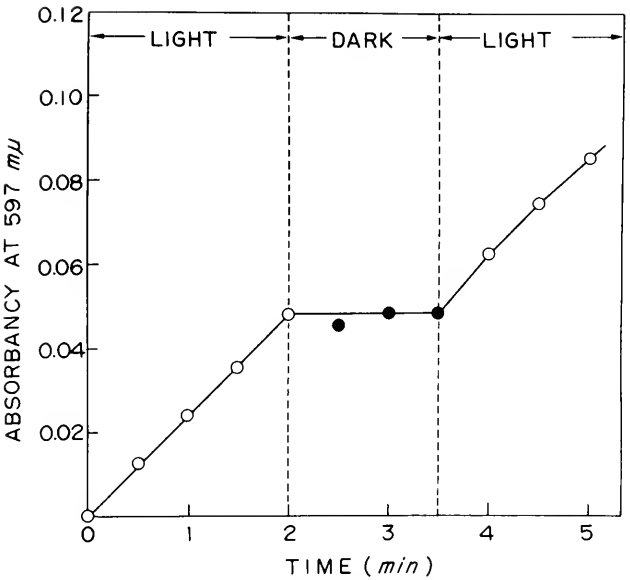

Fig. 1. Time course of photooxidation or reduced plastocyanin with digitonin-treated chloroplasts

oxygen from the reaction mixture by repeated evacuation and flushing with nitrogen resulted in a complete suppression of the oxidation even when illumination.

The rate of photooxidation was maximal at about 5,000 lux. This is considerably lower than the light intensity required for photoreduction of plastocyanin with intact chloroplasts. The pH curve was rather flat with an optimum at pH 8.0 - 8.5.

The reaction was found to be insensitive to various poisons, and there was even a distinct stimulation of the reaction with some of the reagents tested. The stimulation observed in the presence of cyanide, o-phenanthroline and EDTA may be due, in part, to the removal of metal ions from the reaction mixture, since the reaction was highly sensitive to heavy metal ions such as mercury and silver. DCMU at a concentration of 10^{-6} M was completely without effect. PCMB is somewhat inhibitory at a concentration of 10^{-4} M. Chloroplast fragments, which had been heated at 65° for five minutes were incapable of oxidizing the protein. The reaction was markedly stimulated by a rather high concentration of ammonium sulfate, which was previously shown by Bishop et al. to induce a remarkable acceleration of the photooxidation of reduced cytochrome c[16].

All the above-described features of the photooxidation of reduced plastocyanin are in general agreement with those observed for the photooxidation of reduced cytochrome c. A notable difference between the two photooxidizing systems, however, was discovered when digitonin-treated chloroplasts were fractionated with ethanol into a chlorophyll containing fraction and a soluble fraction. Using a similar fractionation procedure, Nieman et al. demonstrated the necessity of a soluble factor, designated Factor 2 by them, for the photooxidation of reduced cytochrome c by the chlorophyll bearing fraction[17]. We have confirmed this observation (Table I). However, the capacity for photooxidation of reduced plastocyanin was not appreciably affected by the ethanol-treatment and the addition of the soluble factor was without any effect on the activity of the ethanol-precipitated fraction (Table I). Evidently, the photooxidation system of reduced plastocyanin requires no additional soluble factor.

TABLE I

Effect of Soluble Factor on the Photooxidation of Reduced Plastocyanin and Cytochrome C by the Ethanol-Precipitated Fraction

Electron donors	Addition		μmole oxidized / mg chl. hour
Reduced plastocyanin	None		48
Reduced plastocyanin	Soluble factor	0.4 ml	50
Reduced cytochrome c	None		3
Reduced cytochrome c	Soluble factor	0.2 ml	15.6
Reduced cytochrome c	Soluble factor	0.4 ml	25.6

On the other hand, it was discovered that plastocyanin can replace the soluble factor in accelerating the photooxidation of reduced cytochrome c by the ethanol precipitated fraction. The reaction rate is increased by the addition of plastocyanin to the level of photooxidation of reduced plastocyanin with the same preparation. This stimulating effect of plastocyanin occurs by a mechanism different from that with ammonium sulfate, since the acceleration observed in the presence of both substances was far higher than the sum of the rates observed when they were added separately (45.6 with plastocyanin alone, 39.1 with 1.3 M ammonium sulfate, and 142 μmoles of reduced cytochrome c oxidized per mg chlorophyll per hour with plastocyanin and ammonium sulfate).

These findings strongly suggest that Factor 2 of Nieman et al. may be plastocyanin. Presumably in the photooxidation of reduced cytochrome c by the digitonin-treated chloroplasts, plastocyanin acts as an intermediary electron carrier which is first oxidized by the photooxidizing system of chloroplasts and then reduced by the reduced cytochrome c.

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EFFECTS OF PLASTOCYANIN ON THE PHOTOREDUCTION OF INDIGO CARMINE, FMN AND NADP IN THE PRESENCE OF ASCORBATE AND DPIP

Further investigations were carried out to test the effect of plastocyanin in various oxidation reduction reactions of chloroplasts. First, it was found that the rates of the Hill reaction with several electron acceptors were significantly accelerated by the presence of a minute amount of plastocyanin. With the Hill oxidants of rather high redox potential, such as cytochrome c and DPIP, the observed acceleration may be explained by the simple assumption that plastocyanin functions as an intermediary electron carrier in the reaction. It could be rapidly reduced by the illuminated chloroplasts and then transfer its electron to the Hill oxidants with high efficiency(8).

In addition, the effect of plastocyanin on the photoreduction of reagents with redox potentials lower than zero, such as indigo carmine, FMN and NADP, was also studied. In this series of experiments, chloroplasts prepared from fresh leaves of Brassica campestris (Komatsuna) were used. They showed a rather low rate of Hill reaction with indigo carmine as the electron acceptor. A significant high rate of photoreduction of indigo carmine, however, was obtained upon the addition of a substrate amount of ascorbate and catalytic amount of 2, 6-dichlorophenol indophenol. This is in accordance with the previously published results of Vernon and Hobbs with chloroplasts from other plant species(18). Now, the further addition of plastocyanin to such reaction mixture resulted in a striking rise in rate of reduction of indigo carmine as shown in Fig. 2. The degree of acceleration was a function of the amount of plastocyanin and saturation was reached at about 0.5 mμmole of plastocyanin added, thereby indicating that the protein functioned catalytically. Plastocyanin itself was entirely inactive in catalyzing the photoreduction of the dye in the absence of chloroplasts.

It will be seen from Table II that the activity for reduction of indigo carmine per unit of chlorophyll decreased both on washing and hypotonic treatment of the chloroplast. It is most probably due to a loss of a soluble factor from the particulate system. The effectiveness of plastocyanin in stimulating the reaction rate, however, was much more enhanced after these treatments. Almost the same rate of photoreduction, on a chlorophyll basis, was obtained with the three fractions in the presence of plastocyanin. It was inferred, therefore, that the chloroplasts requires plastocyanin as an essential component of the photoreduction of indigo carmine in the presence of ascorbate and DPIP. The data in Table II also show that plastocyanin is somewhat effective on the slow photoreduction of indigo carmine in the absence of the ascorbate-DPIP couple. Previously, Vernon and Hobbs noticed that the photoreduction of indigo carmine by chloroplasts was markedly stimulated on addition of the supernatant fluid(18) This result may be ascribed to the possible presence of plastocyanin in the soluble faction.

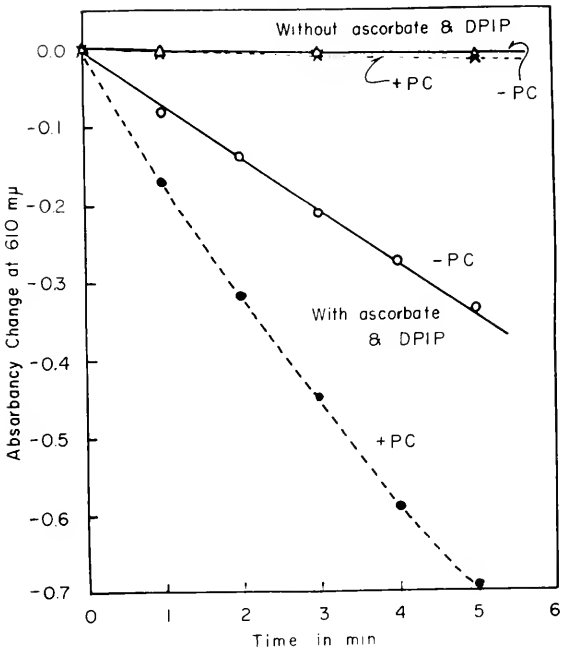

Fig. 2. Effect of plastocyanin on the photoreduction of indigo carmine by Brassica Chloroplast with and without ascorbate and DPIP

TABLE II

Effect of Plastocyanin on the Photoreduction of Indigo Carmine by Leaf Homogenate, Whole Chloroplasts and Broken Chloroplasts

Fractions	μmole IC reduced per mg chl. per hr.			
	With ascorbate + DPIP		Without ascorbate + DPIP	
	- PC	+ PC	- PC	+ PC
Leaf homogenate	22.5	47.5	0.20	0.54
Whole chloroplast	16.9	51.5	0.22	0.52
Broken chloroplasts	9.3	50.1	0.37	0.56

The data presented in Fig. 3 show that the degree of acceleration induced by the addition of a constant amount of plastocyanin was almost unchanged in the absence or in the presence of widely varied concentration of DPIP. This fact suggests that the role of plastocyanin in the photoreducing system of the

dye is essentially different from that of DPIP, transferring electron between ascorbate and the oxidizing site of chloroplasts.

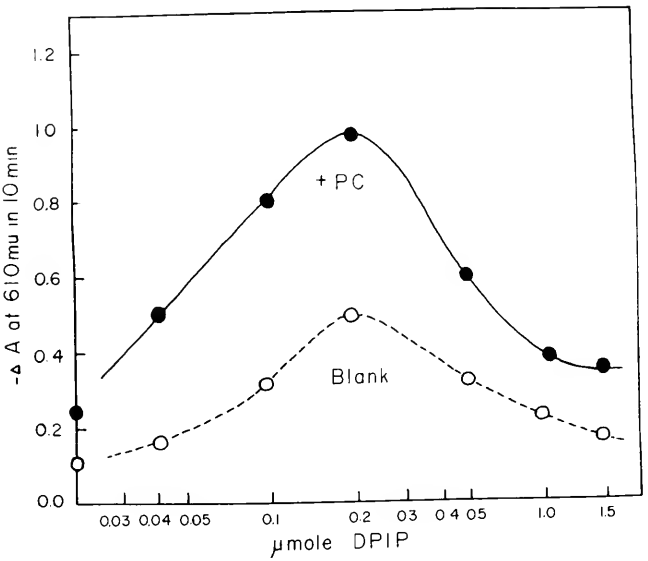

Fig. 3. Effect of plastocyanin on photoreduction of indigo carmine in the presence of various concentration of DPIP

As will be seen in Table III, plastocyanin also stimulates the photoreduction of other low potential substances, such as FMN and NADP in the presence of ascorbate and DPIP.

TABLE III

Effect of Plastocyanin on the Photoreduction of FMN and NADP in the Presence of Ascorbate-DPIP Couple

Electron acceptor	DCMU	Ascorbate + DPIP	μmole reduced mg chl. hour - PC	+ PC
FMN	-	-	19.8	25.7
FMN	-	+	4.6	19.7
NADP	-	-	133	141
NADP	+	-	0	0
NADP	-	+	43	78
NADP	+	+	43	68

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EFFECT OF PHOTOREDUCTION OF NADP BY THE DIGITONIN-TREATED CHLOROPLASTS

Plastocyanin was also found to be effective in stimulating the photoreduction of NADP (Hill reaction), when digitonin-treated chloroplast fragments, which had lost most of the activity in question, were used. The addition of a catalytic amount of plastocyanin resulted in partial recovery of the lost activity. A complete reactivation was obtained at a concentration as low as about 10^{-8} mole per 3 ml of the reaction mixture (Table IV). The stimulating effect observed on addition of an aqueous extract of chloroplast to the digitonin-treated chloroplasts may be also due to the presence of plastocyanin in the extract.

TABLE IV

Effect of Plastocyanin on NADP Photoreduction by Digitonin-treated Chloroplasts

Preparation	Addition (mole)	μmole reduced mg chl hour
Untreated chloroplast	-	118
Digitonin-treated Chloroplast	-	0
	0.13×10^{-8}	4.9
	0.33×10^{-8}	7.8
	0.65×10^{-8}	10.5
	1.30×10^{-8}	11.1
	1.95×10^{-8}	11.0

PPNR is necessary for the reduction of NADP, no photoreduction of NADP is observed when PPNR is omitted even in the presence of plastocyanin. Addition of the following substances could not replace the role of plastocyanin in stimulating the reaction in question; FMN, PMS, vitamin K_3, ascorbate, ferri- and ferrocyanide, benzoquinone, and cytochrome c. The Rhus blue protein, another non-autooxidizable form of copper protein (kindly provided by Dr. Omura[19]), was also without effect. The degree of stimulation due to plastocyanin depends on the light intensity. The effect was not very significant at light intensities lower than 7,000 lux. At this intensity the reaction rate without addition of plastocyanin reaches saturation. With added plastocyanin, however, the reaction rate increases with further increase in light intensity up to 40,000 lux. The plastocyanin-induced photoreduction of NADP is sensitive towards the inhibitors of the Hill reaction, such as DCMU and o-phenanthroline. This fact indicates that the observed change involves the Hill reaction activity including the oxygen evolving system.

In concluding, plastocyanin is another necessary factor which participates in the photochemical activity of chloroplasts, namely, photooxidation of reduced cytochrome c, photoreduction of low redox potential substances in the presence of ascorbate and DPIP, and the Hill reaction with NADP as electron acceptor. The removal of plastocyanin from chloroplasts by various treatments resulted in the loss of these activities which are recovered more or less on addition of plastocyanin. From the experimental facts described above, it may be inferred that this substance is an electron carrier between the two postulated photochemical reactions of the photosynthetic mechanism, although the final decision must await further investigation.

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EVIDENCE FOR THE ROLE OF SEVERAL QUINONES IN THE ELECTRON TRANSPORT SYSTEM OF CHLOROPLASTS

R. A. Dilley, M. D. Henninger and F. L. Crane

PROPERTIES OF CHLOROPLAST QUINONES

Present evidence indicates that there are four compounds of the plastoquinone type in spinach chloroplasts [1]. In addition, there occurs α- β- and γ-tocopherylquinones and vitamin K_1 [2] [3]. Hereafter, the plastoquinones will be abbreviated PQ-A-B, etc; and the tocopherylquinones α- β-TQ, etc. Table 1 lists chromatographic, spectrophotometric, and melting point data of these quinones. The PQ-C and -D data represent the best preparations to date and further purification may be possible.

The four plastoquinones appear to be widely distributed in the plant kingdom. They have all been found in a variety of species including mono- and dicotyledon types and in the green algae Chlamydomonas reinhardi.

Table 1

Properties of Spinach Chloroplast Quinones

Quinone	R_f *	Absorbance Maximum mμ in ETOH	$E^{1\%}_{1\ cm}$ at λmax.	Isobestic Points mμ	M. P.
PQ-A	0. 74	255	246	276, 233	44° C
PQ-B	0. 78	255	202	276, 233	35° C
PQ-C	0. 49	262	96	283, 232	Oil
PQ-D	0. 40	262	55	290, 226	Oil
α-TQ	0. 37	261, 269	414	282, 232	Oil
β-TQ	0. 33	261	430	280, 229	Oil
γ-TQ	0. 25	258	430	279, 230	Oil
Vit. K_1	0. 80	241, 261 249, 269		253, 281	Oil

* Thin layers (250 μ) of silica gel G, chloroform as solvent.

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Separation and Identification of Quinones

Thin-layer chromatography has been of tremendous value in the separation and identification of naturally occurring quinones. In addition to being a rapid means of getting high resolution and handling relatively large quantities of material, this method allows the simultaneous determination of the oxidized and reduced forms of plastoquinones [4]. Preparative strip chromatography is easily accomplished using the thin-layer technique, thus allowing isolation of measurable quantities of compounds which occur in very small amounts.

PHOTO-INDUCED CHANGES OF CHLOROPLAST QUINONES

A study was conducted on the effect of light on the concentration of the oxidized form of α-TQ, PQ-(A+B), and PQ-D in spinach chloroplasts. Thin-layer chromatography was used to isolate the quinones from an acetone extract of the chloroplasts [3]. The conditions of the experiments are given under Table II. Although the incident light intensity was 1600 foot candles, the suspensions were so thick that the chloroplasts may be considered as exposed to a lower light intensity. Table II shows the results of a representative experiment. It is seen that low intensity white light results in an increase in the concentration of α-TQ and PQ-(A+B).

Table II

Light and NADP-Induced Changes in Chloroplast Quinone Concentrations

	α-TQ umoles		PQ-D umoles		PQ-(A+B) umoles	
	dark	light	dark	light	dark	light
1	0.55	1.1	1.3	1.3	6.4	8.1
1 + NADP	0.60	1.6	1.3	2.5	7.4	5.6

Fresh spinach chloroplasts (prepared according to Jagendorf and Avron [5], chlorophyll assayed by method of Arnon [6]) corresponding to 50 mg chlorophyll, 0.05 M tris pH 8.0, 0.01 M NaCl, 7.5 umoles NADP, and distilled water to make final volume 200 ml. Light intensity 1600 foot candles.

The presence of NADP in the reaction mixture resulted in a marked increase in α-TQ and PQ-D levels but a decrease in PQ-(A+B). Friend and Redfearn have also shown a decrease in the concentration of oxidized PQ-A in the presence of added NADP [7]. Other experiments revealed that these light and NADP-induced changes were inhibited by o-phenanthroline at 10^{-4} M, and that a dark incubation period following the light exposure resulted in about the same quinone levels as in the dark control. Experiments are presently underway to study the stoichiometry of the changes in α-TQ,

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α-TQH_2, and α-tocopherol levels under various conditions.

RESTORATION OF ELECTRON-TRANSFER REACTIONS IN EXTRACTED CHLOROPLASTS

In conjunction with studies of the redox state of quinones under various conditions, extraction and restoration experiments provide information regarding the site of action of the naturally occurring quinones.

The results of restoration experiments depend strongly on the solvent used in extracting the lyophylized chloroplasts. Extraction with n-heptane or petroleum ether removes a major portion of PQ-A but very little of the other neutral lipids of interest. On the other hand, acetone removes 90% chlorophyll, 100% PQ-A and PQ-B, 60% PQ-C and D, and 20% or less of the tocopherylquinones [1]. Hence, it is expected that wide variations in results would occur between laboratories using the various extraction methods. In addition to extracting a large amount of the quinones and chlorophyll, the acetone treatment results in an apparent alteration of the binding of the chlorophyll remaining in the chloroplast as evidenced by a shift in the absorption spectrum peak from 678 mμ to 668 mμ (in aqueous suspension).

Table III

Restoration of Photoreductive Activities by Quinones

Quinone	Added μmoles	Acceptor: Water to NADP[1, 4]	DPIP-AA to NADP[2, 4]	Cyt. c[1, 4]	Ferricyanide[3, 4]
Extracted Chloroplasts	none	6.6	7.8	24.	290
Dried Chloroplasts	none	28.	22.	240.	640
PQ-A	0.026	3.0	-15.	- 6.0	600
PQ-B	0.026	30.	14.	15.	530
PQ-C	0.013	4.2	27.	30.	500
PQ-D	0.013	- 0.6	-17.	- 15.	580
PQ-A+D+C	as above	23.	4.2	--	---
PQ-C+D	as above	2.4	- 5.4	72.	240
α-TQ	.005	2.4	-10.	120.	320
β-TQ	.009	0.6	-10.	96.	840
γ-TQ	.001	16.	17.	120.	650

(1) Assayed by the method of Keister et al [8]
(2) Assayed by the method of Vernon and Zaugg [9]
(3) Assayed by the method of Henninger et al [10]
(4) μmoles reduced/hr/mg chlorophyll

Table III shows the effect of adding back the various quinones to acetone extracted spinach chloroplasts. Following extraction there is a decrease in the rates of reduction of ferricyanide, cytochrome c and NADP, but on a chlorophyll basis there is an increase in the photoreduction of indophenol. On a dry weight basis there is a decrease in all the reductase activities.

It is apparent that there is considerable variation in the extent of stimulation of a particular photoreduction by the various quinones.

The photoreactions are inactivated by heating the chloroplast suspension to 100° C for two minutes.

Two general statements can be made about the restoration of photoreductase activities by plastoquinones and tocopherylquinones as shown in Table III. First, none of the quinones stimulate all activities. Secondly, the tocopherylquinones tend to be effective in restoration of certain activities at much lower levels than the amounts of plastoquinones required for restoration of activity. It may also be noted that the tocopherylquinones occur at much lower concentrations in vivo. Ferricyanide reduction is the least selective process and is stimulated to some extent by all quinones except α-tocopherylquinone. β and γ-tocopherylquinones as well as a mixture of PQ-C + PQ-D are also effective for restoration of cytochrome c reduction. Purified PQ-C is also slightly active in this system. The ascorbate dependent NADP reduction is stimulated by γ-tocopherylquinone, PQ-B and PQ-C. In contrast to this the reduction of NADP from water requires the addition of PQ-A + PQ-C + PQ-D on the one hand or PQ-B alone on the other [11]. The combined effects of more than one quinone are consistent with observations of Trebst of more than one quinone site [12] in the electron transport system of chloroplasts.

After acetone extraction there is the appearance of a new type of activity, namely a photooxidation of reduced cytochrome c or NADP in the presence of certain added quinones. This type of activity is illustrated by negative values shown in Table III. In the experiments illustrated in the table where negative values are shown, addition of quinone in the dark leads to a partial reduction of cytochrome c or NADP. When the system is exposed to light, the reduced acceptor is oxidized. This type of activity is best seen when PQ-A or PQ-D is added to the acetone-extracted chloroplasts in the NADP system. Similar effects can be shown when reduced NADP is added at the start with subsequent oxidation occurring in the light. These dark reduction and photooxidation processes are readily reversible. After photooxidation in the light the NADP can be shown to return partially to the reduced form when the system is returned to the dark. It would appear that the addition of certain quinones makes a pool of reducing power available for reduction of NADP in the dark, and that the electron flow is reversed in light to regenerate the pool of reducing power with consequent oxidation of the $NADPH_2$.

The photoreduction of both NADP and cytochrome c has been shown by Keister et al [9] to require the presence of an enzyme, photosynthetic

pyridine nucleotide reductase (PPNR). The restoration of cytochrome c reduction by ∝-tocopherylquinone and by the PQ-C + PQ-D mixture also requires addition of PPNR as does the PQ-A + PQ-D + PQ-C dependent reduction of NADP. The photooxidation of reduced NADP which occurs when PQ-A is added to extracted chloroplasts is also stimulated by addition of PPNR.

These results lead us to the proposal that there are several sites for quinone function in chloroplasts which show considerable specificity with regard to quinone type. Further experimentation will be necessary to determine the site of function of each of these quinones.

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THE PATHWAY OF METMYOGLOBIN AND NADP REDUCTION BY ILLUMINATED CHLOROPLASTS

H. E. Davenport

It has been shown[1] that the "methaemoglobin reducing factor" of Davenport, Hill and Whatley[2] is in its purified form[3] identical with the "photosynthetic pyridine nucleotide reductase" (PPNR) of San Pietro and Lang[4]. More recently the name ferredoxin[5] has been proposed for this same leaf protein and will be adopted in what follows.

When a comparison was made of the catalytic activities of purified preparations of ferredoxin in promoting the photochemical reduction of nicotinamide adenine dinucleotide phosphate (NADP) on the one hand, and sperm whale metmyoglobin on the other, it was found that the relative rates of these two reactions, measured under identical conditions, may show wide variations. The variability did not appear to be related to the source or method of preparation of the ferredoxin but rather to the source and pretreatment of the chloroplasts or grana used in the assay mixtures. The garden pea (Pisum sativum) yielded chloroplast material showing the widest variability, and spinach (Spinacea oleracea) the least. Pea chloroplast material was therefore chosen for use in a systematic examination of the causes of the observed variability.

It had been shown earlier[1, 2, 3] that when pea chloroplasts are prepared in isotonic sucrose a single further wash in the same medium suffices to abolish their capacity to reduce NADP or metmyoglobin unless ferredoxin is added back to the reaction mixture. For this reason it was not possible to establish conclusively that ferredoxin is located exclusively in the chloroplast. When such ferredoxin-depleted pea chloroplasts are fragmented in dilute buffer and washed repeatedly in water the pattern of their reactivity towards NADP and metmyoglobin, assayed in the presence of adequate ferredoxin, undergoes a progressive change. In the experiment cited in Table 1 the capacity of the fragments to reduce NADP was diminished by 87% after six washes whereas the reduction rate with metmyoglobin increased by 60%. In attempts to reverse the inactivation of NADP reduction the pooled wash-fluid was subjected to fractionation with ammonium sulphate. A protein fraction, precipitated between the limits 50-66% saturation was found, after prolonged dialysis to remove ammonium sulphate, to be capable of restoring completely the NADP reducing activity of the chloroplast fragments. The addition of this fraction to the assay system was without effect on metmyoglobin reduction.

TABLE 1

The Effect of Successive Water Extractions of Pea Chloroplasts on Their Capacity to Photoreduce NADP and Metmyoglobin in the Presence of a Saturating Amount of Ferredoxin

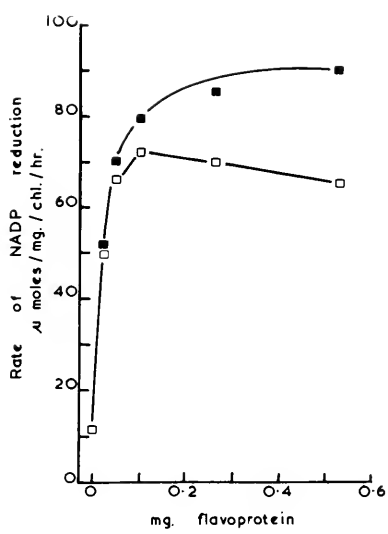
Treatment of Chloroplast Material	Rate of Reduction (μmoles/mg chlorophyll/h)	
	Metmyoglobin	NADP
As prepared in 0·4 M sucrose 0·1 M tris-HCl, pH 7·8	188	87
Extracted with water:		
twice	220	39
three times	265	25
six times	302	11

Reaction mixtures contained (in 3 ml.) chloroplast material equivalent to 0·03 mg chlorophyll; pea ferredoxin 0·15 mg and (in μmoles) NADP 0·5 or metmyoglobin 0·025; tris-HCl buffer, pH 8·0, 150; sodium chloride, 20. Reaction measured as increase in extinction at 340 mμ (NADP) or 582 mμ (metmyoglobin) after successive periods of illumination of 50,000 lux. Gas phase air 20°.

Once it was established that the active material could be extracted from isolated chloroplasts it was found more convenient to prepare it from leaves as a by-product in the routine preparation of ferredoxin either by the method of Davenport and Hill[3] or of Tagawa and Arnon[5]. In either case material precipitated within the ammonium sulphate saturation limits 50-66% was reserved for further purification. The active material was shown by the method of DeLuca, Weber and Kaplan[6] to contain flavin adenine dinucleotide and it will be referred to as "chloroplast flavoprotein."

Ferredoxin and chloroplast flavoprotein were examined as two variable quantities required for the restoration of NADP reduction by protein-depleted pea chloroplasts. Fig. 1 shows that, in a reaction mixture containing a rate-saturating amount of ferredoxin the rate of photochemical reduction of NADP was linearly related to the amount of added flavoprotein until the photochemical system became saturated with respect to this second soluble factor at a reduction rate similar in magnitude to rates obtained with unwashed chloroplasts where ferredoxin is the only soluble factor needed. The converse type of experiment where ferredoxin was the variable factor is shown in Fig. 2.

Independent evidence that a factor normally retained within the chloroplast is involved in NADP reduction was obtained by the selective inhibition of this second factor by phenyl mercuric acetate (PMA) in situ. It has been shown[3, 4] that mercurial compounds inactivate ferredoxin; their inclusion in the assay mixture would not, therefore, be expected to inhibit metmyoglobin and NADP reduction differentially. In order to localize the action of the heavy



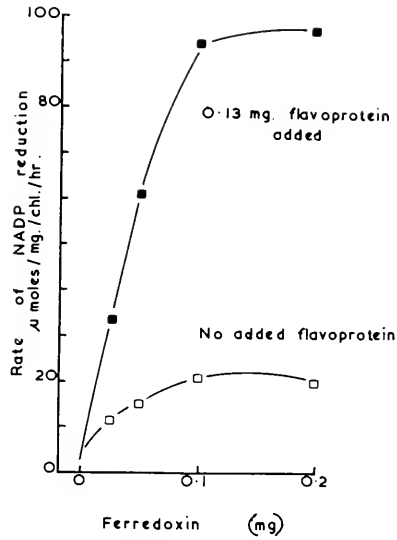


Fig. 1. (Left) Activity of pea-leaf flavoprotein fraction in restoring the capacity of protein-depleted pea chloroplast fragments to photoreduce NADP in the presence of saturating concentration of ferredoxin. Experimental conditions as for NADP reduction in Table 1 except that each cell contained pea chloroplast fragments extracted six times with water and equivalent to 0·025 mg chlorophyll. □, Experimental; ■, corrected for dark oxidation.

Fig. 2. (Right) Activity of pea ferredoxin in mediating NADP photoreduction by protein-depleted pea chloroplast fragments. □, With no further additions; ■, in the presence of a saturating amount (0·13 mg) of a pea-leaf flavoprotein fraction. Experimental conditions as Fig. 1.

metal pea or spinach chloroplasts prepared in isotonic sucrose were pretreated for 5 min. in sucrose solution containing 5×10^{-4} M PMA and then washed twice in sucrose solution. Such treated chloroplasts when compared with others which had undergone a similar series of washes in sucrose alone were found, when supplemented by ferredoxin, to retain unchanged their capacity to reduce metmyoglobin. By contrast their NADP reducing capacity was impaired to a large but variable extent. (Table 2)

It was reported earlier[1] that ferredoxin-catalyzed reduction of NADP is stimulated by the presence in the reaction mixture of a phosphate-accepting mixture consisting of ADP, Mg++ and orthophosphate. The pattern of this stimulation in a reconstituted system of washed pea chloroplasts supplemented by ferredoxin and chloroplast flavoprotein is shown in Fig. 3. Phosphorylation of ADP was shown to accompany the stimulated reaction rate.

TABLE 2

The Effect of Treating Intact Pea Chloroplasts with Phenyl Mercury Acetate on Their Capacity to Photoreduce NADP and Metmyoglobin

Chloroplast Treatment	Rate of Reduction (μmoles/mg chlorophyll/h) Metmyoglobin	NADP
Untreated	168	60
Treated with PMA	170	20
Inhibition (%)	0	66

Reaction mixtures as Table 1 except that chloroplasts contained 0·045 mg chlorophyll.

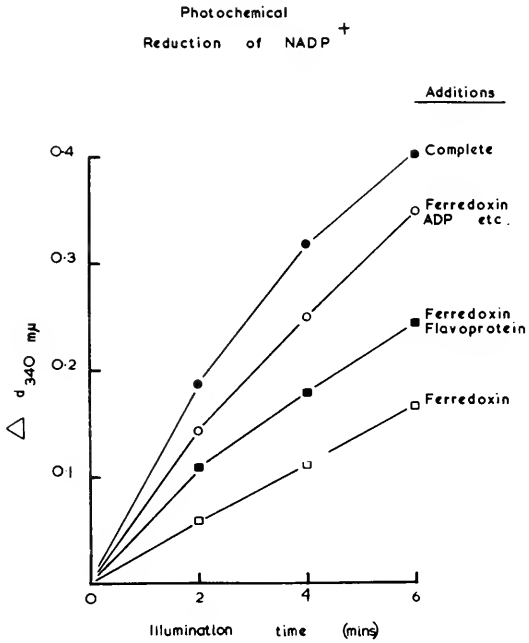


Fig. 3. Effect of ferredoxin, chloroplast flavoprotein and a phosphate accepting system on NADP reduction by protein-depleted pea chloroplast fragments.

Reaction mixtures contained (in 3 ml) four times extracted pea chloroplast fragments equivalent to 0.015 mg chlorophyll and (in μmoles) Tris HCl pH 8, 150; NADP, 0.5; NaCl, 20. Where indicated ferredoxin, 0.15 mg, flavoprotein 2.0 mg, and (in μmoles) ADP, 0.5; Na_2HPO_4, 5.0; $MgCl_2$, 10 were added. 30,000 lux, 20° air.

The results described appear to be consistent with the reduction sequence shown diagrammatically as Fig. 4. The electron transport pathway leading to the reduction of NADP is that proposed by Tagawa and Arnon[5] and the diagram shows also the position of metmyoglobin in the reduction sequence. This interpretation is supported by the further observation that in a soluble system both ferredoxin and flavoprotein are required for the dark reduction of metmyoglobin by NADPH. Lazzarini and San Pietro[7] have described a similar reaction where cytochrome c replaced metmyoglobin and where the flavoprotein was identified as transhydrogenase. Attempts to detect transhydrogenase activity in the pea chloroplast flavoprotein by the method of Keister and San Pietro[8] have been unsuccessful and this negative finding would appear to preclude the reaction sequence postulated by Lazzarini and San Pietro for the photochemical reaction.

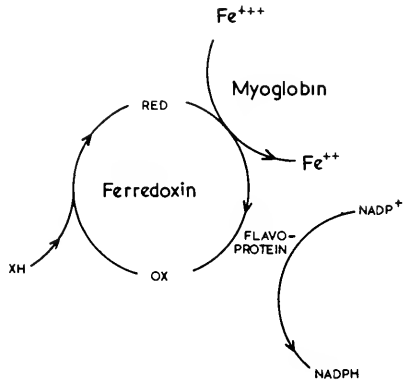


Fig. 4. Diagrammatic representation of metmyoglobin and NADP reduction by illuminated chloroplasts

The pea leaf flavoprotein has properties similar to those of the NADPH specific diaphorase extracted from spinach chloroplasts by Avron and Jagendorf[9].

In the original isolation of ferredoxin from plant material[1, 2] the metmyoglobin reducing activity was used as a rapid and convenient assay method for the protein without reference to the nature of the natural hydrogen acceptor in the plant cell. The results recorded above suggest that the method may still be of value as a general assay method uncomplicated by a requirement for a second catalytic factor. Some experiments with a partially purified preparation of Clostridium ferredoxin (a gift of Dr. J. E. Carnahan) support this suggestion.

Fig. 5 shows that the Clostridium protein is highly active as a "methaemoglobin reducing factor" but is markedly inferior to pea ferredoxin as a "pyridine nucleotide reductase."

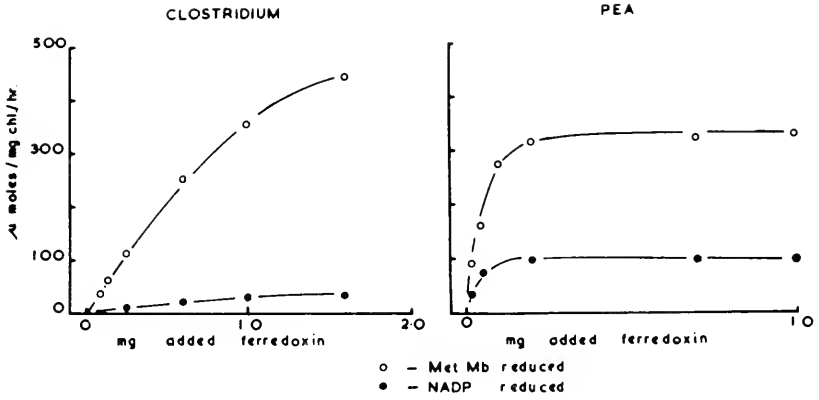


Fig. 5. A comparison of the activities of pea and Clostridium ferredoxins as catalysts of NADP and metmyoglobin reduction by illuminated pea chloroplasts

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ON THE PARTICIPATION OF CYTOCHROME f IN PHOTOSYNTHETIC ELECTRON TRANSPORT

Giorgio Forti, Maria Luisa Bertolè and Bruno Parisi

Cytochrome f was first isolated by Hill and Scarisbrick[1] from higher plants, and obtained in highly purified form from parsley[2]. Evidence for the occurrence of this pigment in a large number of photosynthetic tissues has been presented[2], as well as for its being present only in green tissues[2].

In order to investigate the participation of cytochrome f in the photosynthetic reactions of isolated chloroplasts and grana, we have studied a new method for the purification of cytochrome f, utilizing the excellent initial extraction procedure reported by Davenport and Hill[2], with only one modification. This consisted of the addition of the non-ionic detergent Triton X 100 (at the concentration of 1% v/v) to the ethanol-ammonia extraction solvent. The new method, to be published elsewhere[3], involves further ammonium sulfate fractionation and column chromatography on Sephadex dextrans. A highly purified preparation was obtained, showing a ratio O.D. at 422 mμ to that at 278 mμ of 2.8. The absorption spectrum, as well as the reduced minus oxidized difference spectrum is identical to the one reported by Davenport and Hill[2]. The purity of the present preparation seems slightly higher, as indicated by the higher ratio of absorbancy at 422 mμ to absorbancy at 278 mμ. The most purified preparation still retains catalase activity, as reported by Davenport and Hill[2]. Whether this activity is due to contamination or is an intrinsic property of cytochrome f is not clearly established at this moment.

REACTIONS OF CYTOCHROME f WITH ISOLATED CHLOROPLASTS

Cytochrome f is reduced by chloroplasts or grana in the light, as previously reported[4], and ATP formation is coupled to this reaction with a $P/2e^-$ ratio of 1[4].

Recent experiments showed that the photoreduction of cyt. f is observed in air as well as in N_2 atmosphere, contrary to our first report that N_2 was needed. Careful washing of the chloroplasts seems to be critical for efficient photoreduction in air. According to current schemes on the function of cytochrome f in photosynthesis, this component should be oxidized by the same photochemical system which is responsible for TPN reduction (chlorophyll a, or system 1 of Duysens and Coworkers[5]), and should be reduced by the photochemical reaction of the "accessory pigments" (system 2[5]). Such a scheme would imply that cyt. f reduction is inhibited by p-chlorophenyldimethylurea (CMU),

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and this is indeed observed[4]; also, that it should be oxidized in the light by chloroplasts, in the presence of photosynthetic pyridine nucleotide reductase (PPNR) and TPN. This is clearly not the case[4]: under no conditions was oxidation of added cytochrome f by chloroplasts or grana observed, in the light or in darkness. Furthermore, the presence of oxidized cyt. f inhibits the photoreduction of TPN by chloroplasts in the presence of rather large amounts of PPNR. This is shown in figure 1. It can be seen that only when

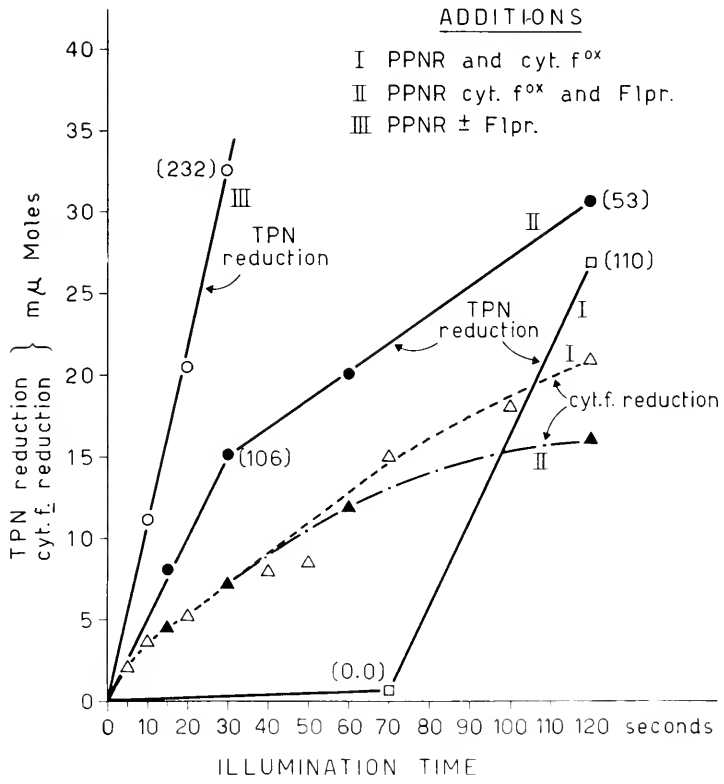

Fig. 1 - TPN and cyt. f photoreduction. Tris buffer 0.05 M, pH 8.0; $MgCl_2$ 0.005 M; ADP 0.003 M; Pi 0.005 M; TPN 10^{-3} M. New-Zealand spinach (Tetragonia) chloroplasts containing 17γ of chlorophyll. Final volume: 1 ml. Additions: cyt. f^{ox} 21 and, respectively 16 mμmoles; flavoprotein 0.9 units (0.9 μmoles of ferricyanide reduced per minute) PPNR 0.05 ml. Light: 20.000 Lux.

all or almost all the cyt. f has been reduced, TPN reduction begins. Under this aspect, cyt. f behaves like ferricyanide[6]. Fig. 1 also shows that the addition of the purified chloroplast flavoprotein (prepared according to Keister et al.[7] prevents the inhibition of TPN reduction by oxidized cyt. f. These observations indicate that cyt. f photoreduction by chloroplasts is competitive with the reduction of TPN. In other words, the same photochemical system (chlorophyll a, or system 1 of Duysens et al.[5]) is providing electrons for the reduction of both TPN and cytochrome f, in contrast with the widely accepted scheme according to which cytochrome f is reduced by the accessory pigment system and oxidized by chlorophyll a. More direct evidence was found to support this hypothesis. The chloroplasts are able to reduce cytochrome f in the dark, utilizing TPNH as the specific electron donor. The ratio of this enzymatic activity to diaphorase and transhydrogenase activity of chloroplasts is shown in table 1. Under the conditions indicated in table 1, the rate of non-enzymatic reduction of cyt. f^{ox} by TPNH is approximately 3% of the enzymatic reaction. It can be seen from the data reported that the ratios of the three activities are comparable for chloroplasts and for the purified enzyme. Furthermore, the observed rates of TPNH-cytochrome f reductase activity of spinach chloroplasts are adequate to account for the observed rates of cytochrome photoreduction, ranging around 40-50 μmoles per milligram of chlorophyll per hour, under light saturation conditions. Higher rates of photoreduction, up to 120 μmoles/mg Chl. per hour, have been observed with Tetragonia chloroplasts.

TABLE 1

Enzymic Activities of Chloroplasts and of Purified Flavoprotein

Activity	Spinach chloroplasts μequivalents/mg chl. h.	Purified flavoprotein μequivalents/mg protein min.
TPNH ⟶ $Fe(CN)_6$	790	48.00 (152)
TPNH ⟶ DPN	8.04	0.93 (290)
TPNH ⟶ cyt. f^{ox}	40.00	3.00

Conditions: Tris buffer 0.05 M, pH 8.0; TPNH 10^{-4}M; Ferricyanide 5×10^{-4}M DPN 10^{-3}M. Cytochrome f^{ox} 10^{-5}M. The number between brackets indicates specific activities as defined by Keister et al.[7].

TPNH-CYTOCHROME f REDUCTASE OF SPINACH

As indicated in table 1, the purified diaphorase-transhydrogenase[7] has a strong cytochrome f reductase activity. Table 2 summarizes the different

activities of the enzyme. It can be seen from table 2 that cyt. f is reduced by the enzyme 3 times faster than DPN, no additions being required. Cytochrome c is not reduced without the addition of an intermediate carrier as FMN or PMS. The enzyme is specific for TPNH as the electron donor, and no activity is observed with DPNH (table 2).

TABLE 2

Activities of the Purified Flavoprotein From Spinach

Substrate	Activity	Addition None	Addition FMN 5×10^{-6}M
TPNH 2×10^{-5}M	$Fe(CN)_6$ reduction	48.8	-
id.	cyt. f reduction	5.9	-
id.	cyt. c reduction	0.06	1.12
id.	DPN reduction	2.24	-
id.	O_2 reduction	0.23	5.0
DPNH 2.7×10^{-5}M	cyt. f reduction	0.00	-
id.	$Fe(CN)_6$ reduction	0.26	

Conditions: Cytochrome f^{ox} 1.64×10^{-5}M; cytochrome c 1.5 mg/ml; Ferricyanide 5×10^{-4}M. Tris 0.05 M, pH 8.0. The concentration of TPNH was kept constant by use of Glucose 6-P dehydrogenase system. The values are expressed in μ equivalents/min. x mg protein.

Preliminary experiments showed that the Ks for TPNH is approximately 4×10^{-6}.

The question of whether the three activities, namely diaphorase, cyt. f reductase and transhydrogenase are due to the same enzyme or to different proteins present in the preparation remains to be established. The cytochrome f reductase could possibly be the same protein as the diaphorase of Avron and Jagendorf[8], and the cytochrome c reductase (requiring the addition of FMN) described by Marrè and Servettaz[9]. These Authors suggested[9] that cytochrome f could be the natural electron acceptor for the enzyme. The properties of the enzyme are being studied.

DISCUSSION

The results obtained suggest an hypothesis on the participation of cytochrome f in photosynthetic electron transport, different from the most widely accepted scheme according to which cyt. f mediates the electron transfer from

water (through the photochemical system 2[5]) to the oxidizing agent formed upon absorption of light by system 1. According to our hypothesis, cytochrome f is reduced by the electrons coming from light-excited system 1 (chlorophyll a) via PPNR, flavoprotein and TPN. Such an hypothesis is supported by the following evidence: (a) Reduced cyt. f is not photooxidized by chloroplasts in the presence of PPNR and TPN, with or without the further addition of purified flavoprotein, in the presence of CMU to prevent photoreduction by water[4]. (b) The chloroplasts contain TPNH-cyt. f reductase activity (table 1), which is due to a flavoprotein purified together with the TPNH diaphorase-transhydrogenase of Keister et al.[7]. The ratio of diaphorase, transhydrogenase and cytochrome f reductase activities of chloroplasts closely resembles the ratio between these activities found with the purified enzyme. Finally, (c), oxidized but not reduced cyt. f inhibits the photoreduction of TPN by the system chloroplasts-PPNR, in spite of the fact that the rate of TPN reduction by the same system is much higher than the rate of cyt. f reduction (see fig. 1). The inhibition is largely prevented by the addition of purified flavoprotein (or also by the addition of large amount of chloroplasts, which contain the enzyme), as shown in figure 1.

This finding can be explained assuming that cyt. f^{ox} binds to the reduced flavoprotein, which thus would be prevented from reacting with TPN. The following reaction scheme can explain the reported observations:

(1) 2PPNRH + flavoprotein $\longrightarrow$ 2PPNR + fl.pr. H_2

(2) fl.pr. H_2 + 2cyt. f^{ox} $\longrightarrow$ fl.pr. + 2 cyt. f^{red}

(3) fl.pr. H_2 + TPN $\longleftarrow$ fl.pr. + TPNH

Reactions (3) from right to left and reaction (2) account for the TPNH-cytochrome f reductase of the purified flavoprotein.

It should be noted that our evidence with extracted cytochrome f is not in agreement with currently proposed schemes[5] in which the primary results of photo-act I is the oxidation of bound cytochrome f. Of course, it should not be expected that added cyt. f would take its ordinary place in the chloroplast electron transport chain, since internal, tightly bound cytochrome is already in place. However it is not unreasonable to expect added cyt. f to donate or accept electrons from the membrane-bound enzyme; as for instance is the case with cytochrome c added to mitochondria.

The failure to see photo-oxidation of added cytochrome f under any circumstances (including the addition of CMU) might possibly be due to its participation in a cyclic electron flow. If so, however, the steady state level of cytochrome f in the cycle would have to remain very close to 100% reduced; which seems most unlikely. In addition, little or no stimulation of ATP formation has yet been observed due to adding reduced cytochrome f, even though the reduction of the oxidized form does support phosphorylation.

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Recent results of Chance et al.[10] indicate that a cytochrome b, rather than cytochrome f, is rapidly oxidized when Chlamydomonas cells are illuminated with short flashes of light of 6943 Å (absorbed by system 1) obtained by means of an optical maser. The oxidation of cytochrome f, observed upon more prolonged illumination, "bears a remote relationship to the photosynthetic apparatus"[10]. These observations are in agreement with our results on the lack of photooxidation of cyt. f by system 1 in isolated chloroplasts.

In conclusion, our results provide the basis and some evidence for the hypothesis that cytochrome f is reduced in the light by chloroplasts by means of electrons coming from system 1[5], through the action of PPNR and a TPNH-cytochrome f reductase present in chloroplasts. This enzyme has been extensively purified together with the diaphorase-transhydrogenase of Keister et al.[7].

Some properties of the new enzymatic activity discovered are described, including a preliminary value of Ks for TPNH of 4×10^{-6}M. It is to be established whether the transhydrogenase and cytochrome f reductase activities can be separated by further purification. The results so far obtained do not allow any speculation on the path of cyt. f photooxidation by chloroplasts: it is possible that the same O_2-requiring, light-requiring system responsible for the oxidation of ascorbate[11] and of quinones[12] might be involved, with the participation of other intermediates.

ACKNOWLEDGMENT

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THE PHOTOSYNTHETIC AND RESPIRATORY SYSTEMS IN EUGLENA GRACILIS

Fulvio Perini

INTRODUCTION

Normal Euglena cells are unique among microalgae in lacking chlorophyll if grown in the dark. Dark-grown wild type cells have proplastids and protochlorophyll and after exposure to light form plastids and chlorophyll [1]. During the investigation of this phenomenon, Nishimura [2] found a new cytochrome which was called Euglena gracilis cytochrome 552 or c-552. This haemoprotein had many properties in common with the low molecular weight acidic c-type haemoproteins which were isolated from several genera of algae by Katoh [3]. He postulated the existence of a class of algal c-type cytochromes of high redox potential, possibly connected with photosynthesis. Euglena cytochrome c-552 was included in this class. Additional and more complete work on cytochrome c-552 was carried out by Wolken and Gross [4], who used a different approach and technique. The same workers also discovered another cytochrome in dark-grown wild type and mutant strains. This protein was called Euglena cytochrome 556, although the asymmetrical α-band has its maximum at 558 mμ. Smillie [5] showed the presence of cytochrome c-552 in chloroplasts from Euglena, and the existence of a b-type cytochrome, which he called b_6, like the higher plant counterpart. The result of the present investigation confirmed most of the mentioned findings. The availability of Euglena mutants blocked at various stages of synthesis of the photosynthetic apparatus led to the problem of differentiation between the respiratory and photosynthetic electron transfer systems in this organism. In addition to cytochromes c-552, b-561 (b_6), 556 and a-605, photosynthetic pyridine nucleotide reductase (PPNR) and flavoproteins were also found.

METHOD OF PREPARATION

Cytochromes c-552 and 556

Light-grown wild type cells which had been washed in 0.1 N NaCl + 0.05 phosphate buffer (pH 6.5) were centrifuged at low speed.

The pellet was resuspended in the same kind of buffer mixture as above and kept at -20° C till needed and then thawed. The supernatant liquid after centrifugation was saved, while the sediment was resuspended in a buffered (pH 6.5) mixture of sodium chloride-phosphate and subjected to the same treatment as above. (Buffered salt mixtures used in this investigation are always 0.1 ionic strength in phosphate.) The two supernatant liquids were combined and ammonium sulfate was added to 0.4 saturation, keeping the pH near neutrality. The sediment was discarded and the supernatant liquid dialysed against phosphate buffer (0.02 M, pH 6.8) and added to a column of diethylaminoethyl-cellulose (DEAE). The portion which was not adsorbed could then be added, after a brief dialysis period against the same buffer, to a carboxymethyl-cellulose (CMC). The band from DEAE contained cytochrome 552, which could be eluted with 0.25 ionic strength buffered salt mixture (pH 6.8), and rechromatographed for further purification. Cytochrome 556 was eluted from CMC with 0.05 M phosphate buffer (pH 6.8). Each cytochrome preparation was completely free of other haem compounds.

Cytochrome 556 alone could be prepared in higher yields from etiolated and plastid-less mutants by adjusting the pH of the extracting mixture to 7.5.

A ratio of 300-400 chlorophyll molecules to 1 cytochrome 552 molecule was found even in purified preparations, indicating excellent recovery. The amount of cytochrome 556 was 15-20% that of cytochrome 552.

PPNR

Slightly higher yields of cytochrome 552 were obtained when acetone powders were made, but complications arose in the purification because a brown-red pigment was extracted under conditions of high salt concentration. The highest yields were at pH 8.0. An acetone powder, made from cells which had been exhaustively extracted for soluble cytochromes, as described, was suspended in ammonium sulfate (0.4 sat., pH 8.0). The supernatant liquid after centrifugation was then fractionated with ammonium sulfate,

yielding three fractions: 1) 0.5-0.7 sat. containing a flavoprotein; 2) 0.7-0.9 sat. containing cytochrome c-552; 3) 0.9-1.0 sat. containing PPNR.

PHYSICAL AND CHEMICAL PROPERTIES

Cytochromes c-552 and 556

These have been described in a previous report and by Wolken and Gross. They are summarized in Table 1.

The spectrum of cytochrome c-552 is shown in fig. 1.

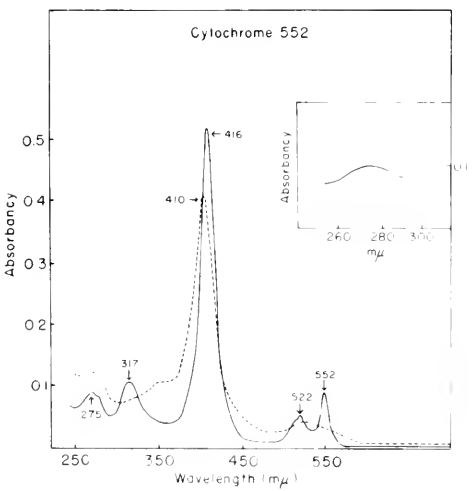

Fig. 1. Absorption spectra of reduced (continuous line) and oxidized (broken line) cytochrome c-552.

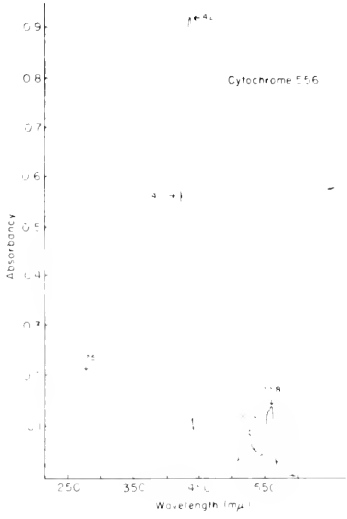

Fig. 2. Absorption spectra of reduced (continuous line) and oxidized (broken line) cytochrome 556.

Cytochrome c-552 is an acidic c-type cytochrome of 13-14,000 molecular weight per haem iron atom, as determined by sedimentation-diffusion, amino acid composition, iron and haem analysis.

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A peptide containing 15 amino acids, obtained after peptic digestion, contains the only two cysteine residues which were found in this protein and the haem. The amino acid analysis indicates an unusually high proportion of acidic and neutral amino acids and three tryptophan residues.

The haem obtained by silver sulfate fission[6] appears to be identical with that obtained from mammalian cytochrome c.

Spectrally, cytochrome c-552 is different from all other algal haemoproteins and more similar to mammalian cytochrome c.

PPNR absorbs in the Soret region at the same wavelength as the maximum of cytochrome c-552, therefore, preparations of this cytochrome which contain PPNR could give incorrect spectral data.

Cytochrome 556 is far more unstable than cytochrome 552 under drastic conditions such as exposure to dilute alkali, acid, and high salt concentrations.

Methylethylketone (pH 2.0) did not remove the prosthetic group from either cytochrome 552 or 556. Treatment with silver sulfate-acetic acid yielded products which were identical with haem c on paper chromatography, the product of fission from cytochrome 556 had a different absorption maximum in acid (haem 556 = 340 mμ; haem 552 and c = 403-4 mμ).

The spectrum of cytochrome 556 (fig. 2) is somewhat like that of higher plant cytochrome f [7] and other algal haemoproteins. The pyridine haemochromogens of cytochrome 556 and haem 556 were different from products obtained from classical c- and b-type compounds.

Preparations of cytochrome 552 which were homogeneous in the ultracentrifuge were obtained. This was not possible with cytochrome 556, however, it was prepared in a highly purified state.

Cytochromes b-561 and a-605

Acetone powders of light-grown cells which had been extracted for soluble cytochrome were examined in a Cary model 14 apparatus provided with the 1462 scattered transmission attachment. This made it possible to obtain difference spectra of suspensions with maxima which appeared to be due to a b-type cytochrome or

TABLE 1

Properties of cytochromes from Euglena

Cytochromes	a-605	c-552	556	b-561
Localization	mitochondria	plastids	mitochondria	plastids
Reduced spectrum	603-5,442-4	552,522, 416	558(554.5) 525,421	561,530, 431
Reduced pyridine haemochromogen	585-90	549	552.5	556
Oxidation-reduction potential (pH 7.0,25°C,mv)	-	370	307	-
Auto-oxidizibility	rapid	none	rapid	slow
Prosthetic group	haem a	haem c	similar to haem c	protohaem
Cyto/300 chl	0.1-0.2	1	0.2	1
Molecular weight	-	13-14,000	17,000	-
Soret/α ratio	-	5.6	6.3	9.0

peroxidase and an a-type cytochrome (fig. 3). See also Table 1. This observation was confirmed by the preparation of pyridine haemochromogens and determination of protohaem a by paper chromatography. Peroxidase assays were negative, save for a small amount of activity due to denatured cytochrome 552. The b-type cytochrome, which was called cytochrome b-561, is probably identical to the b_6 observed by Smillie (5). This protein could not be solubilized and was found to be present in 1:1 ratio to cytochrome c-552.

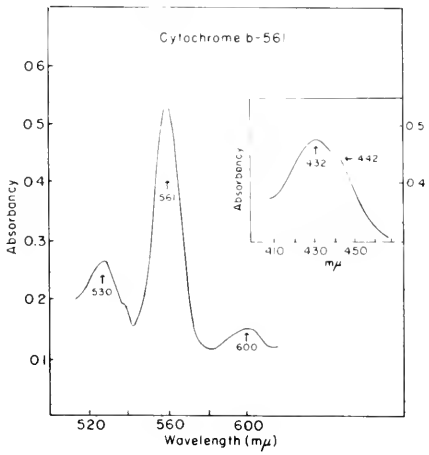


Fig. 3. Reduced-oxidized difference spectra of cytochromes b-561 and a-605.

The autooxidizable compound (the maximum at 600 mμ in fig. 3 is due to autooxidation) identified as an a-type cytochrome was called cytochrome a-605 and was found in all mutant strains and wild type forms of Euglena gracilis in 1:1 ratio to cytochrome 556. It was sensitive to cyanide and to low oxygen tension.

LOCALIZATION AND FUNCTION OF CYTOCHROMES IN EUGLENA

Cytochrome c-552 has been localized in the chloroplasts, whereas cytochrome 556 occurs in small particles, which we believe to be mitochondria. In both cases the procedure used was similar to that used by Smillie (5) for the preparation of chloroplasts from dried cells by suspending them in a mixture of carbon tetrachloride and cyclohexane. The cells were broken in a French press and then fractionated, using solvent mixtures of different specific gravity.

Mutants which have no chloroplasts have no cytochrome c-552, while smaller amounts of the protein were found in a mutant with damaged chloroplasts.

The parallel syntheses of cytochromes c-552 and chlorophyll were followed, and no cytochrome appears to be present in the

first 3-4 hours after illumination of the cell. After 8-12 hours the well-known ratio of 3-400 molecules of chlorophyll/1 molecule of cytochrome c-552 (8) is established, and photosynthetic processes proceed quite well. (See fig. 4 and 5.)

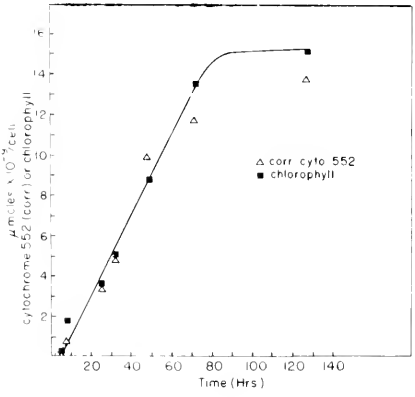


Fig. 4. Kinetics of chlorophyll and cytochrome c-552 formation after illumination of dark-grown wild type Euglena gracilis cells.

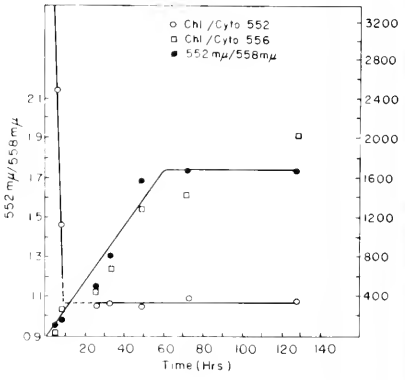


Fig. 5. Relationship between cytochromes c-552, 556 and chlorophyll after illumination of dark-grown wild-type cells.

Cytochrome 556 is present in all mutant strains of Euglena in the same amount as the normal wild type on a cell basis (there are slight differences on a weight basis). No change in cytochrome 556 content was observed after illumination of etiolated cells. A summary is given in Table 2.

Cytochrome a-605 was detected in the mitochondria.

No direct proof was obtained of the presence of cytochrome b-561 in the chloroplasts, probably owing to the instability of its prosthetic group. This cytochrome was found only in photosynthetic forms of Euglena. More than one flavoprotein has been found in Euglena. These have been assigned to the succinoxidase,

TABLE 2

Distribution of cytochromes in different forms of Euglena

Form	Cytochromes	Photosynthetic apparatus	μmoles x 10^{-11}/cell	
			Cyto 552	Cyto 556
Etiolated wild type	556,a-605	proplastid	none	0.75
Light-grown wild type	c-552,556, b-561,a-605	plastid	4.2	0.7
Albino (W_3,W_8)	556,a-605	none	none	0.73
Yellow (Y_3)	556,a-605	detectable	none	0.76
Pale (P_1)	c-552,556, b-561,a-605	incomplete	1.2	0.61

pyridine nucleotide (PN)-oxidase, and to the photosynthetic system. The latter may be the transhydrogenase described by Lazzarini et al. (9)

PPNR

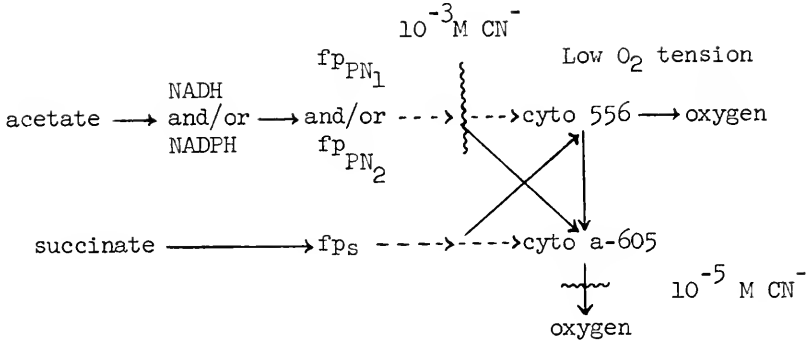
This protein is obtained in oxidized form and has an absorption spectrum with broad maxima at 465-470 and 400-420 mμ.

A ratio of 40 chlorophyll molecules to 1 atom of non haem iron was determined from a chloroplast preparation (10). PPNR was found only in photosynthetic forms of Euglena.

ENZYMATIC ACTIVITIES

The following enzymatic activities have been found in Euglena: 1) succinnic dehydrogenase; 2) NADPH (reduced nicotinamide adenine dinucleotide phosphate)- and NADH (reduced nicotinamide adenine dinucleotide)-haemiprotein reductase (non-specific, probably catalyzed by a transhydrogenase, NADPH is much better coenzyme); 3) TPN-reductase (via ferrodoxin, i.e. PPNR, and flavoprotein, may be the same as 2)); 4) succinoxidase or PN-oxidase in presence of 95% CO or CO_2/5% O_2. Reduced cytochromes c-552 and 556 very poorly replace cytochrome c in a cytochrome oxidase system. In the presence of cyanide (10^{-4} M) and low oxygen tension, cytochrome 556 is oxidized more rapidly than cytochrome a-605. On readmission of oxygen, cytochrome 556 is first reduced by the remaining substrate, and then oxidized, whereas cytochrome a-605 exhibits a net oxidation which is more rapid than under semi-anaerobic conditions. When oxygen becomes available, cytochrome 556 is either not used, or participates in processes before cytochrome a-605, in the respiratory system. This investigation was made with fresh washed cells suspended in buffer, using the scattered transmission attachment of the Cary.

The pathways, as shown on the next page, explain the resistance of Euglena to some poisons and its resistance to anaerobic conditions (11). In some cases the pathway going through a cytochrome 556-type may predominate, and a-type cytochromes may be present in very small amounts, if at all. These mechanisms may explain the unusual resistance to some metabolic poisons by many microalgae and protista, and the difficulty in finding any evidence for a classical cytochrome oxidase.



Steps indicated by broken lines are hypothetical and have not been investigated.

No suggestion can be advanced from this research about how or where cytochromes c-552, b-561 and PPNR function in the photosynthetic apparatus.

Acknowledgments

This investigation has been supported in part by the National Institutes of Health grants and by the C. K. Kettering Foundation.

The author is grateful to Professor M. D. Kamen and Dr. J. Schiff for their valuable help and interest in this work.

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IV. ENHANCEMENT STUDIES: GAS EXCHANGES

ENHANCEMENT

Jack Myers

The photosynthetic phenomenon which has come to be known as enhancement arose, not from any theoretical insight, but simply from the attempt to understand an experimental anomaly. On the long wavelength side of the in vivo absorption spectrum, the quantum yield drops rapidly even at wavelengths (i.e., 690 mμ) where chlorophyll a absorption is still high.[7,14] Study of the anomaly led Emerson and co-workers to the discovery of enhancement.[5] When imposed upon a constant background of shorter wavelength illumination, long wavelength light (690 mμ) gave a higher rate of photosynthesis and a quantum yield approaching maximum. Further, it turned out that a significant intensity and maintained rate of photosynthesis was needed in the short wave background. The phenomenon is not one of "catalytic" or triggering action, not a secondary light effect, but an intrinsic character of photosynthesis. Hence a more useful description of enhancement becomes the following: a light beam of wavelength λ_1 and another beam of properly chosen wavelength λ_2, when presented together, elicit a rate of photosynthesis greater than the sum of the rates when presented separately.

THE HYPOTHESIS

Enhancement observed between alternated light beams and other more direct lines of evidence, which are discussed in other papers of this symposium, support the most obvious hypothesis for enhancement: that two photoreactions (I and II) are necessary for green plant photosynthesis. The spectral character of actinic light by which the two photoreactions are partially distinguishable implies participation of two pigment systems (1 and 2, following the convention of Duysens and Amesz[3]). So far we have not discovered in any plant a wavelength at which quanta can be injected exclusively into either pigment system and thence to the corresponding photoreaction. However, we can identify wavelength regions in which quanta are absorbed in excess by either one of the two pigment systems.

Jack Myers

One of the first concerns in the study of enhancement was the character of pigment systems 1 and 2 or, experimentally, spectra of the enhancement phenomenon. A comparative approach was possible because of availability of algal groups with diverse pigment complements. While the exploration has not been completed, it is possible to anticipate two general results. (1) In any one alga there are two spectral regions λ_1 and λ_2. A combination of wavelengths chosen between the two will show enhancement. A combination of wavelengths within either region will not show enhancement.[17,26] (The latter statement is recognized as in conflict with certain data.[2,12]) (2) Even without precise data on in vivo absorption, qualitative conclusions can be drawn from the spectra of λ_1 and λ_2 about the character of pigment systems 1 and 2. System 2 is associated with high absorption of accessory pigments phycoerythrin, phycocyanin, fucoxanthin, chlorophyll b, and the special in vivo chlorophyll a-673.[2,8,9,11] System 1 is associated with the bulk of chlorophyll a. There is uncertainty about the role of carotenoids. The salient features of these conclusions were foreseen by Emerson in his speculations on the role of accessory pigments.[4]

EXPERIMENTAL DIFFICULTIES

Study of enhancement has been complicated by experimental difficulties. Use of narrow bands of monochromatic light places increased demands on sensitivity and precision of measuring photosynthesis. Even as used by Emerson and co-workers the Warburg method is marginal in sensitivity. The bare platinum electrode developed by Blinks and Haxo[14] provides adequate sensitivity and rapidity but measures only relative changes in net rate of oxygen evolution and has its own special limitations.[18,25] The mass spectrometer can provide unequivocal measurement but requires considerable calculations to find readout results.[16]

Further experimental difficulties arise from even minor nonlinearities in light intensity curves at either of the wavelengths used. One of these is the Kok effect which will be discussed later. A second kind of nonlinearity can be introduced by approach to light saturation.[1]

Comment should be made on the special question of whether the enhancement phenomenon is in any way related to differences in light-saturated rate of photosynthesis at different wavelengths as reported by McLeod[22] for Chlorella. Our attempts to confirm these results so far have been uniformly negative.[27] We have found special experimental problems but no simple explanation of the differences between our results and McLeod's. However, we do not now

feel obliged to include explanation of wavelength dependence of light-saturation in current theory of enhancement.

CHARACTERISTICS IN ANACYSTIS AND CHLORELLA

Quantitative Expression

Enhancement may be expressed in several different ways. Without debating the merits of several possible ways I shall simply state my own rationale. The enhancement effect at any wavelength (λ_1 or λ_2) is seen most clearly when observed on a large excess of the complementary wavelength region.

Suppose that we observe a rate of oxygen evolution p_1 at intensity I_1 of wavelength λ_1 and a rate p_2 at intensity I_2 of complementary wavelength λ_2 (using the subscript notation previously defined). When I_1 and I_2 are presented together we observe a rate p_{12} which is larger than $p_1 + p_2$. We can now express a ratio or gain factor E referred to either wavelength alone:

$$E_1 = \frac{p_{12} - p_2}{p_1} \qquad (1)$$

$$E_2 = \frac{p_{12} - p_1}{p_2} \qquad (2)$$

Equations (1) and (2) are permitted by the important finding both in Chlorella[1,23] and in Anacystis[17] that E_1 and E_2 are functions of the ratios between, but not the absolute values of, I_1 and I_2 or p_1 and p_2.

Quantitative Relations

Fig. 1 shows E_1 vs. p_2/p_1 (A) and E_2 vs. p_1/p_2 (B) from experimental data on Chlorella[23,26] and Anacystis.[17] There are several aspects of importance. Each curve shows an initial linear segment and a final plateau at which E_1 or E_2 reaches a maximum value. Curvature between the two regions may be attributed to several causes, one of which is clearly evident. Because of great differences in absorption between 650 and 710 mμ in Chlorella or between 620 and 700 mμ in Anacystis, it is impossible to maintain a constant ratio of I_1 and I_2 (or p_1 and p_2)

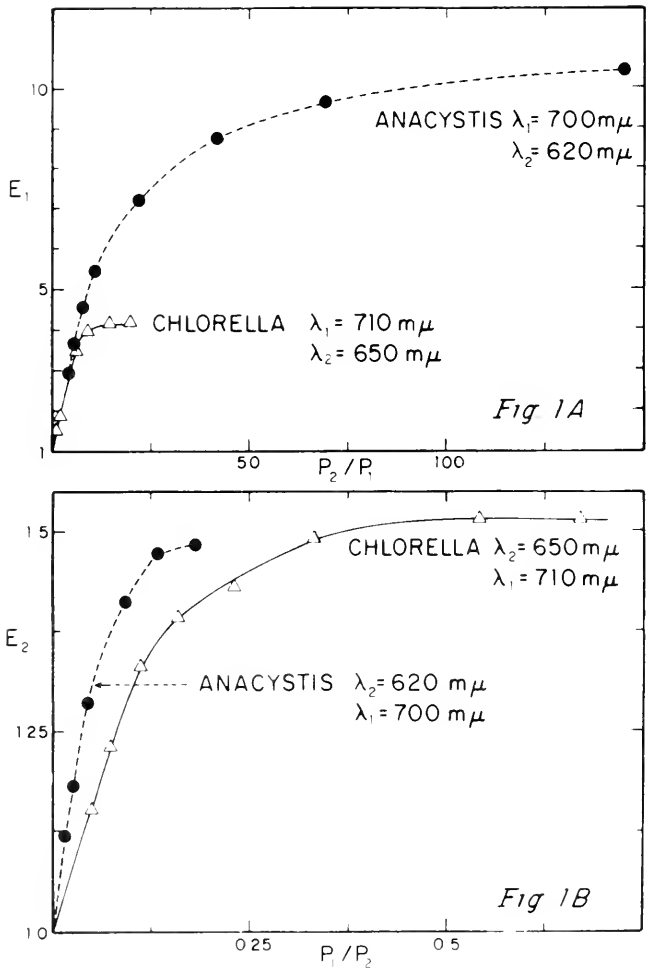


Fig. 1. Enhancement, E_1 at λ_1 (A) and E_2 at λ_2 (B), vs. intensity of complementary wavelength expressed as $p_2/\overline{p_1}$ or p_1/p_2. Data for Chlorella were obtained from thin films of low absorption; data for Anacystis were taken from experiments with thick films of high absorption.

across an algal film of any appreciable absorption. The I_1/I_2 ratio necessarily varies considerably within the film. The curves show the requirement for most instructive study of spectra. Enhancement in the λ_1 region (E_1) is seen most clearly in an excess of λ_2, practically a very high p_2/p_1 ratio. Enhancement in the λ_2 region (E_2) is seen most clearly in an excess of λ_1, practically requiring only a rather modest p_1/p_2 ratio.

Enhancement Spectra

Fig. 2 and 3 present enhancement spectra obtained under conditions meeting the requirements noted above. In Chlorella (Fig. 2) the λ_1 and λ_2 regions intersect at about 685 mμ. The λ_2 region has been associated with chlorophyll b and chlorophyll a-673.[9,11] The question of role of carotenoids does not seem answerable. Fig. 3, taken from the recent work of Jones[17] on Anacystis, shows spectra for two kinds of cells of widely differing pigmentation. Cells grown under usual tungsten illumination (W) and with a "normal" phycocyanin/chlorophyll ratio show a λ_2 region of about 530 to 655 mμ. There are two segments to the λ_1 region, $<$ 530 and $>$655 mμ. If cells are grown under red light $>$ 660 mμ (practically under BCJ dark room lamps with necessary addition of a small amount of clear tungsten illumination), they show a much reduced chlorophyll. Such cells (curve R, Fig. 3) show decreased E_1 and increased E_2 enhancements together with broadening of the λ_2 region. From the E_1 enhancement at 460 to 510 mμ a case can be made for carotenoid participation in system 1. However, the case cannot be made from casual inspection of the curves. These are not action spectra and enhancement cannot be related to absorption by any one pigment alone; it can be related only to absorption ratios between different active pigments.

Though there are important differences from the spectrum reported by Blinks[2] for Anabaena, the curves for Anacystis confirm his finding that the λ_1 region may have two segments, one in the blue and the other in the far red.[13] Hence it is clear that the λ_1 region is related to pigment absorption and not to some peculiar character of long wavelength.

DISTRIBUTION OF LIGHT QUANTA

I turn now to the problem of distribution of light quanta between the two photoreactions or, in the casual language of Kok, how the carburetor of photosynthesis works. Enhancement in intact-cell photosynthesis is regarded as a tool for study of quanta distribution as viewed by the over-all rate. Suppose that

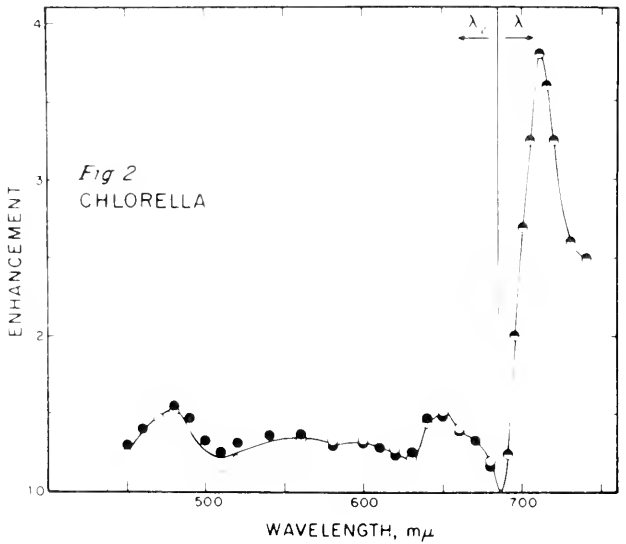


Fig. 2. Enhancement spectrum of Chlorella [23,26] in which E_1 for λ_1 and E_2 for λ_2 were obtained under conditions of excess background of complementary wavelength.

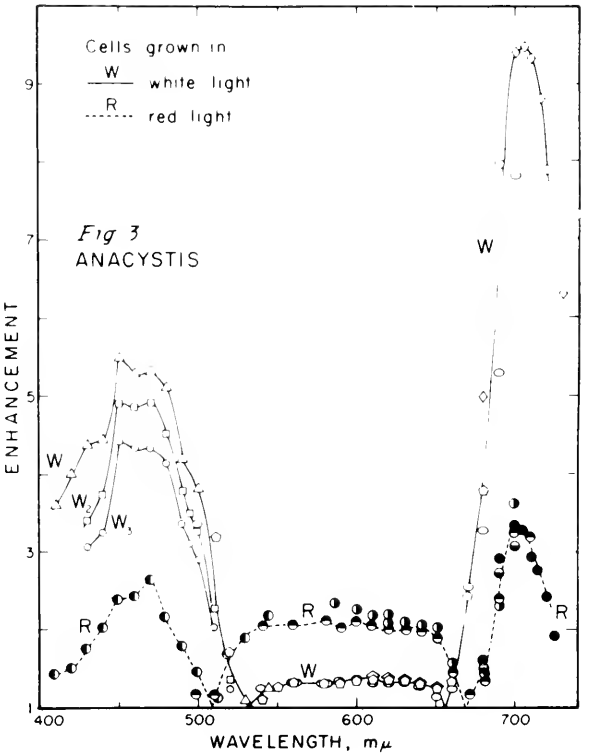


Fig. 3. Enhancement spectra of Anacystis[17] similar to Fig. 2 and measured under conditions, chosen to eliminate the Kok effect.

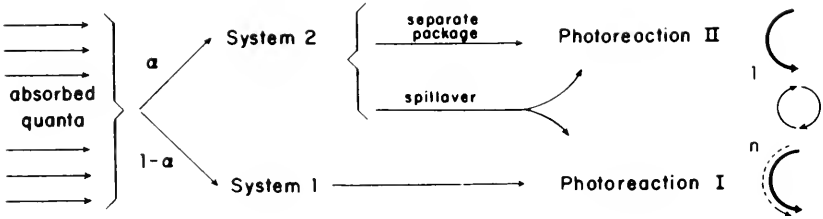
two light beams of wavelength λ_1 and complementary wavelength λ_2 show enhancement. Then in at least one of the beams alone the quantum yield must be less than maximum simply because quanta are distributed in excess to one of the two photoreactions. Addition of the second beam permits the use of quanta previously wasted and gives a more than additive increase in oxygen evolution. We interpret enhancement by this simple extension of the two-photoreaction hypothesis.

A framework for discussion of the distribution problem is presented in Fig. 4. In order to supply the intermediates for thermal reactions, it is supposed that photoreactions I and II are required in a stoichiometric ratio, n. One can visualize, for example, that cyclic phosphorylation may place extra demand on I and a value of $n > 1$. Hence we cannot now specify a value and must leave n as an unknown in subsequent analysis.

One point of distribution of quanta is determined by absorptions of the two pigment systems. We define by α the fraction of total absorbed quanta which are absorbed by pigment system 2. A second possible point of quanta distribution occurs within the pigment systems in transfer of quanta to the terminal photoreceptors (such as P700 for photoreaction I). In this transfer we can imagine two possible arrangements. In the first and simplest we imagine that the two pigment systems are arranged, perhaps spatially, so that there is an invariant transfer within each system to its specific photoreceptor. Such an arrangement, suggested by Duysens and Amesz,[3] we have called the separate package model. We have also imagined a second possible arrangement called the spillover model. In this we envision that quanta absorbed by system 2 (largely composed of accessory pigments) are transferred preferentially to the photoreceptor for II, but also may be transferred to the photoreceptor for I if II is "full." Reasons for considering the spillover model have been discussed previously.[26] It is not a novel concept, having been considered, at least in principle, by W. A. Arnold, by Hans Gaffron (personal communications), and probably also by others.

In short, there are now two alternate models for quanta distribution. The separate package model supposes that the distribution at any wavelength is governed only by absorption into the two pigment systems. The spillover model requires a two-stage distribution system, the first by pigment absorption ratios and the second in energy transfer between pigments. Actually the two models merely describe limit possibilities. For example, if energy transfer can occur between system 2 and photoreaction I

Fig. 4



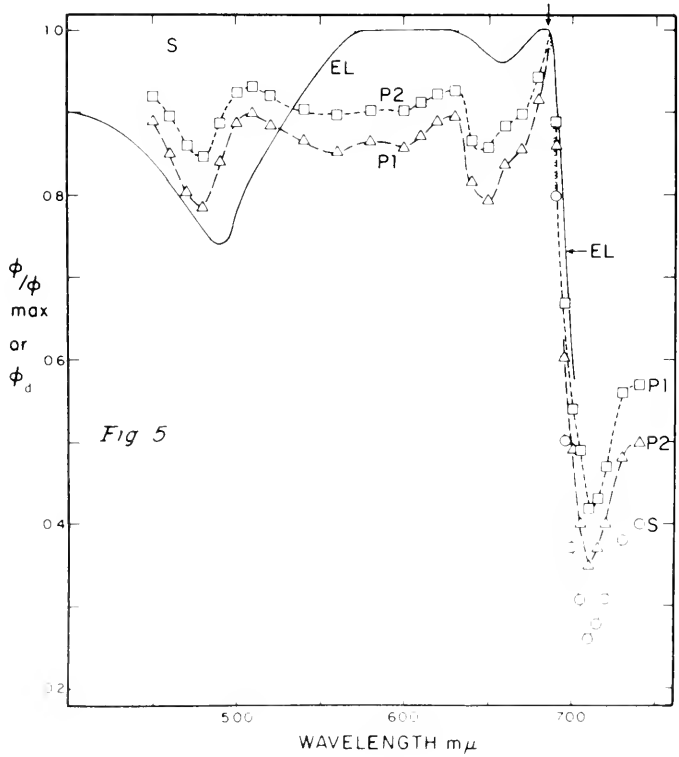


Fig 5

Fig. 5. Quantum yield comparisons. See text.

but with lowered efficiency, then a description would fall somewhere between the two models. The two models have quite different consequences in restrictions which they require in terms of composition of the two pigment systems, quantum yield, and the nature of enhancement.

From the simple hypothesis on the nature of enhancement there can be derived the relations shown in Table 1 for each of the models. In relating values of E, α, and n to quantum yield we use ϕ_d to denote the relative quantum yield (ϕ/ϕ_{max}) as affected by quanta distribution. Values for ϕ_d and E, derived independently in terms of α and n for each of the models, permit evaluation of ϕ_d in terms of E_1 or E_2 and n. The analysis presented is only an extension of that of Bannister and Vrooman[1] but with some change in conventions.

In search for choice between the two models we have attempted various analyses of enhancement data for comparison with pigment absorption or quantum yield data. Partly because of uncertainty in the value of n, we have not found any test which leads to an unequivocal answer. I shall cite one such test with respect to quantum yield in Chlorella. Plotted in Fig. 5 are values of relative quantum yield vs. wavelength. Curve EL presents the data of Emerson and Lewis.[7] The other curves are for ϕ_d calculated as in the last column of Table 1 from the E_1 and E_2 enhancement values of Fig. 2. Curve S describes predictions from the spillover model. Curves P describe predictions from the separate package model for values of n = 1 (P_1) and n = 2 (P_2). The spillover model predicts constant and maximum quantum yield across the λ_2 region to 685 mμ. The separate package model predicts a maximum quantum yield at only one wavelength, the crossover point 685 mμ where E_1 and E_2 approach unity. We ignore the region below 560 mμ because of uncertainties due to carotenoid absorption. The experimental curve beyond 570 mμ is flat except for a 4% dip at 650-660 mμ. The separate package model requires a much greater dip for any reasonable value of n. There is no uncertainty that the separate package model requires a less than maximum quantum yield whenever $E > 1$. There is uncertainty whether the data of Emerson and Lewis have sufficient precision to discriminate between the two models.

THE KOK EFFECT

Since enhancement in monochromatic beams has been studied only at relatively low light intensities there has been a recognized

Table 1. Relations for Rates of Photoreactions I and II, Enhancement, and Quantum Yield Derived for the Models of Fig. 4

	For λ_1 and λ_2 Alone			For λ_1 and λ_2 on Excess Complementary Background			
	Rate I	Rate II	ϕ_d	Rate I	Rate II	E_1 or E_2	ϕ_d
λ_1, with Excess to System 1							
Separate Package	$n\alpha_1'$	α_1'	$(n+1)\alpha_1'$	$1-\alpha_1'$	$\frac{1-\alpha_1'}{n}$	$\frac{1-\alpha_1'}{n\alpha_1'}$	$\frac{n+1}{nE_1 + 1}$
Spillover	$n\alpha_1$	α_1	$(n+1)\alpha_1$	$\frac{n}{n+1}$	$\frac{1}{n+1}$	$\frac{1}{(n+1)\alpha_1}$	$\frac{1}{E_1}$
λ_2, with Excess to System 2							
Separate Package	$1-\alpha_2'$	$\frac{1-\alpha_2'}{n}$	$(1+\frac{1}{n})(1-\alpha_2')$	$n\alpha_2'$	α_2'	$\frac{n\alpha_2'}{1-\alpha_2'}$	$\frac{n+1}{n+E_2}$
Spillover	$\frac{n}{n+1}$	$\frac{1}{n+1}$	1.0	$n\alpha_2$	α_2	$(n+1)\alpha_2$	1.0

α' is used in place of α for the separate package model. Distinction is necessary since the value assigned is not the same for both models, i.e., $\alpha \neq \alpha'$.

α_1 and α_2 subscripts are used to distinguish between values for the λ_1 and λ_2 regions. α_2 is used in place of the β of Bannister and Vrooman.

E_1, E_2, ϕ_d, and n are defined in the text.

possibility that the effect might be associated with a respiratory anomaly, with changes in respiratory oxygen uptake rather than with changes in oxygen production. In at least one alga, Anacystis, the question has been answered unequivocally by the mass spectrometer experiments of Hoch, Owens and Kok.[16] We interpret their results as follows: (1) there are effects of low light intensity on respiration, most pronounced in the λ_1 region; (2) these are of such a nature that they may obscure or decrease enhancement effects as observed in terms of net oxygen production; and (3) the enhancement effect is not caused by changes in respiratory oxygen uptake.

The important experiments of Hoch et al.[16] provided an explanation of the Kok effect[19] which we also observed in Anacystis. Our studies with the Blinks-Haxo electrode were less definitive in that they measured only changes in net oxygen production. However, their greater precision and rapidity led to results which further broaden the interpretation.[18]

Fig. 6 demonstrates the Kok effect in Anacystis as a sharp break in slope of the light intensity curve at very low intensity. The effect is observable by our measurements only at wavelengths of the λ_1 region. Further, curves A, B, and C for the different wavelengths extrapolate to a common intercept. Following the results of Hoch et al., we attribute the high initial slopes (curves A, B, C) to the combined effects of decreasing rate of oxygen uptake and an increasing rate of oxygen production. The lower final slopes represent only increasing rate of oxygen production superimposed upon a lowered, but now constant, rate of oxygen uptake.

Fig. 7 shows the change in rate of net oxygen evolution in response to intensity at 700 mμ when added to a constant background intensity of chosen wavelength. The reference curve A (dark background) is taken from the previous set of measurements without correction for a measured 10% decay in response between the two sets. Curve E, with constant 700 mμ background, shows only one linear segment extrapolating to zero but otherwise corresponding to the second slope of curve A. The respiratory decrease has been saturated and its effect wiped out by the 700 mμ background. Conversely, curves F and G on 620 mμ background have an initial slope essentially the same as the slope of curve A and prolonged to an extent dependent on background intensity. Curves F and G demonstrate enhancement between the constant 620 mμ background and the varying 700 mμ. As expected, both curves approach the same final slope, common also to curves A and E, as the 620 mμ

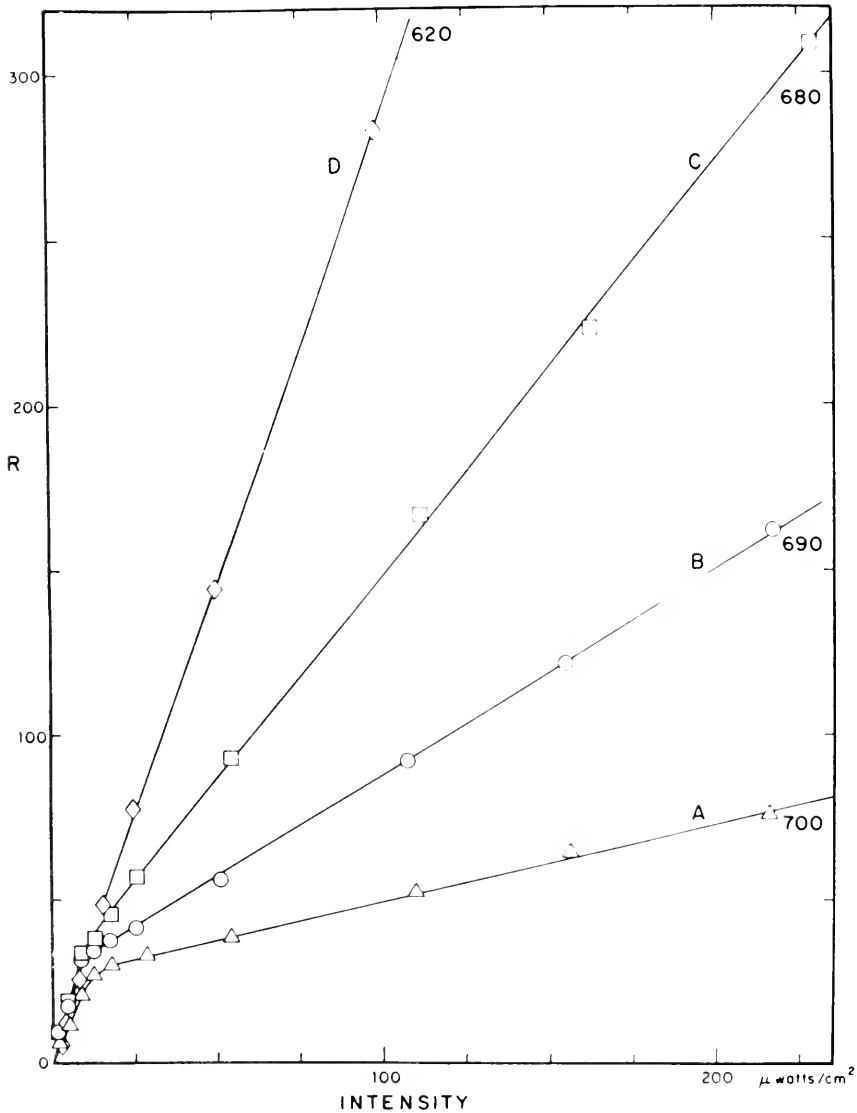


Fig. 6. Net rate of apparent oxygen production R, vs. light intensity for Anacystis[18] obtained with a thick algal film.

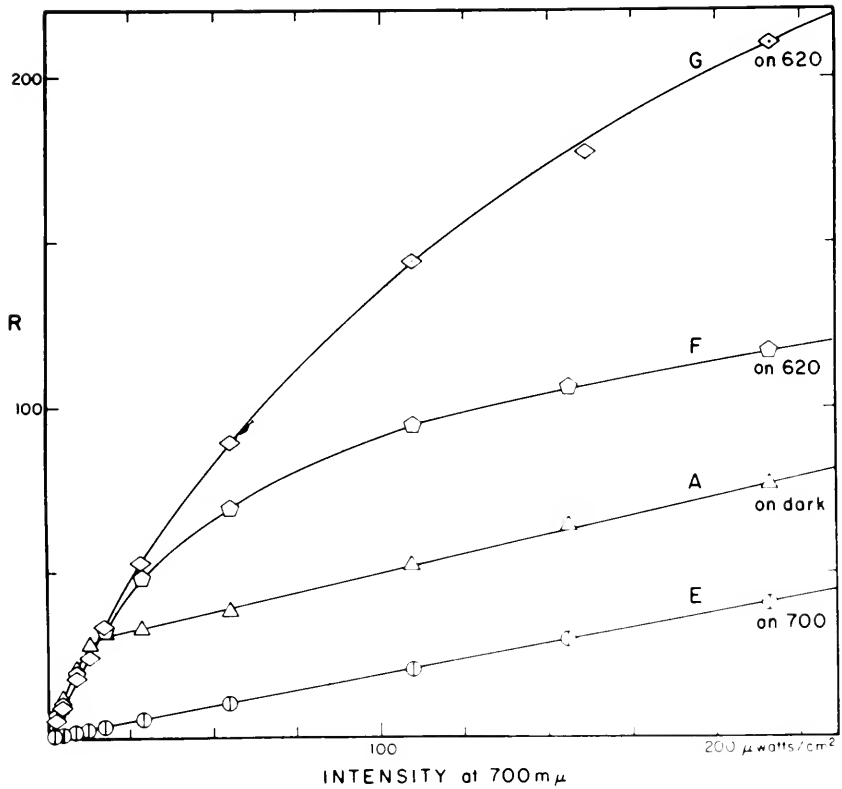


Fig. 7. As Fig. 6 but R vs. intensity of 700 mμ when superimposed on backgrounds of constant intensity at the wavelengths indicated on each curve.

background becomes limiting.

The most important finding is that the initial slope of curve A is identical to the initial slope of the enhanced curves F and G and that the ratio between the first and second slopes of curve A is identical to the maximum value for enhancement (E_1). Similar results were obtained at wavelengths 680 and 690 mμ. In short the initial slope is not enhanceable. At very low intensities the quantum yield, reckoned as increase in net oxygen evolution per absorbed quantum, is maximum.

We interpret the result in terms of the Hill and Bendall[15] model: that the two photoreactions are separated by an electron transport system. The simplest explanation is that P700 is, in effect, a competitor of oxygen. We have proposed[18] that the competition is direct in the sense envisioned by Lundegardh[21]: that in Anacystis the same system serves both the transport of "photosynthetic" electrons from photoreaction II to P700 and also part of the transport of "respiratory" electrons to oxygen. We recognize, however, that competition could be much less direct. Hoch et al.[16] have suggested a decreased "respiratory" electron flow in response to a lowered ADP concentration caused, in turn, by extra cycling of photoreaction I.

The Kok effect occurs even more dramatically in Anabaena variabilis[17] but appears in Chlorella pyrenoidosa only as a minor nonlinearity.[26] Where it does appear there are a number of important consequences. (1) If the effect occurs in Chroococcus as it does in Anacystis and Anabaena, then (as previously noted[16]) the high quantum yield values obtained by Emerson and Lewis[6] on Chroococcus at 660 to 700 mμ probably are in error. (2) The effect can completely obscure the enhancement phenomenon or even give rise to the "negative" enhancement noted in early work.[8,11] The difficulty can be eliminated by referring enhancement to an increment rather than to a single value on the light intensity curve. (3) Unless the light intensity curve is carefully determined, the effect may easily be mistaken for a very early light saturation in the λ_1 region.[11] (4) If there are pathways of electron transport common to both respiration and photosynthesis, then it would seem that in vivo absorption changes would yield more instructive information if studied as changes in response to an increment of light rather than (or in addition to) light-dark changes.

Jack Myers

CONCLUDING REMARKS

This discussion has not attempted to consider and explain all observations on enhancement and related phenomena. It has omitted consideration of a quite different model which can account for enhancement and respiratory effects of light in Porphyridium.[10] The discussion has been based upon an intensive study of two algae, Anacystis and Chlorella. With both algae we experienced early difficulties under which we failed to see enhancement at all or obtained inconsistent results. Once these difficulties were recognized and solved we never thereafter failed to see a consistent behavior of enhancement. We plan to extend the same rationale to algae of other phyla.

Finally, comment should be made on possible limitations which accompany the very great advantage of a comparative approach. Availability of algal phyla with different pigment complements has made it possible to reach the generalization that there are two photoreactions in some way associated with two pigment systems. For example, if we had only data on Chlorella we would still be concerned whether enhancement was in some way peculiar to long wavelength. However, the algal phyla also differ in cell organization. It seems doubtful a priori that characteristics of photosynthesis will differ between the phyla only in spectral absorption of their pigments. Ability of the green plant to use efficiently a broad portion of the solar spectrum seems even more remarkable in the face of the problem of running two photoreactions at some fixed ratio. One cannot avoid the question why, of the several pigment systems developed in the course of evolution, the chlorophyll a-chlorophyll b system became a preferred choice.

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EMERSON ENHANCEMENT EFFECT AND TWO LIGHT REACTIONS IN PHOTOSYNTHESIS

Govindjee

Dedicated to the memory of late Professor Robert Emerson

During the 1940's Robert Emerson discovered[1,2] that the quantum efficiency of photosynthesis, plotted as a function of wavelength, drops appreciably long before one reaches the long-wave limit of absorption by chlorophyll a. This decline is referred to as the "red drop." Recently it has become clear that in green cells, this "drop" begins when the form of chlorophyll a, absorbing at the longer wavelength, becomes the prime absorber. Persistence of a certain photosynthetic activity to wavelengths up to 740 mμ could be interpreted either in terms of fractional absorption by the short-wave form of chlorophyll a (Chl a 670) extending into the far red, or by ascribing to the "long-wave form" of chlorophyll a, the capacity of bringing about complete photosynthesis but with a very low yield. Excitation of the "long-wave form" of chlorophyll a alone in green cells certainly is insufficient to bring about all photochemical steps involved in photosynthesis with a high yield. In the case of the red and blue-green algae, the drop occurs at shorter wavelengths[3,4] apparently, when chlorophyll a takes over the role of the main absorber from the phycobilins. It seems that all (or almost all) chlorophyll a in red and blue-green algae has the same photochemical function as do the long-wave forms of chlorophyll a in green plants.

In the photosynthesis laboratory at Urbana, a long series of experiments[4-8] revealed that by exciting one of the "short-wave pigments" (chlorophyll b and Chl a 670 in green algae; phycoerthrins and phycocyanins in red and blue-green algae; fucoxanthol, chlorophyll c and Chl a 670 in diatoms), simultaneously with the long-wave form of chlorophyll a, the "red drop" could be avoided. Simultaneous excitation of two "pigment systems" permits the plants to use efficiently the energy absorbed in the "long-wave" form of chlorophyll a. This synergistic effect is referred to as the "Emerson enhancement effect". Studies

of this effect have given the first evidence for the now widely postulated[8-14] hypothesis of two photochemical systems participating in photosynthesis. The enhancement effect has been studied by manometry[2, 4-8], polarography[11-17], mass spectrometry[18-20] and by the radiocarbon tracer technique.[21] Its finding both in oxygen evolution and in CO_2 uptake shows that it is characteristic of photosynthesis as a whole. Experiments on the Hill reaction[22-24] suggested the involvement of the two pigment systems in this process as well. Further evidence for the existence of two such systems has come from difference spectroscopy[10b, 25-28], electron spin resonance studies[29-31], studies of fluorescence[32-36], and luminescence (afterglow)[37], absorption spectroscopy[38, 39], as well as studies on mutants[40], from flashing light experiments[22,41], and from biochemical investigations.[42]

This paper summarizes some of our recent work on the "red drop" and the enhancement effect in whole cells and in chloroplast preparations.

I. INVESTIGATIONS ON THE "RED DROP" IN PHOTOSYNTHESIS

A. Algae Pioneer studies of Emerson and coworkers showed the occurrence of "red drop" in several algae (Chlorella[1,2], Anacystis[8c], Porphyridium[4,8c], and Navicula[8c,43]). At 20°C, the decline in the quantum yield of photosynthesis in Chlorella begins at 680 mμ, and the yield is halfway down to zero at 696 mμ. This decline is affected by several factors: (a) Presence of appropriate background light ("enhancement effect"). In this case, the decline may disappear altogether, (see figure 1).

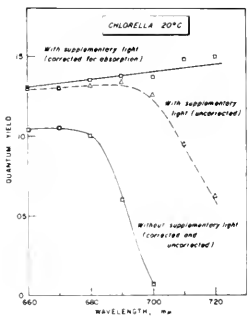

Figure 1 Quantum yield of oxygen evolution as a function of wavelength in thick suspensions of Chlorella (corrected and uncorrected for absorption); (data from Emerson).

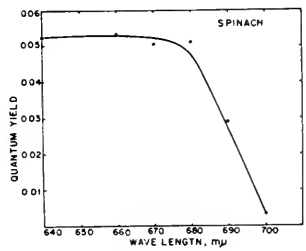

Figure 2 Quantum yield of oxygen evolution in spinach chloroplasts, as a function of wave - length (ferricyanide + catalytic amount of 2,6 DCPIP). (Data from Bakri and Govindjee).

(b) Temperature. Lowering the temperature shifts the beginning of the decline to longer wavelengths.[2] At 10°C, the quantum yield[44] starts declining only at 690 mμ and is halfway down at 730 mμ. (c) Growing the algae in "earth extract" (an undefined organic medium). This too, shifts[7] the beginning of the decline to longer wavelengths and causes the enhancement phenomena to disappear. (d) Growing the algae in glucose medium[45] does not shift the beginning of the decline appreciably, although there is a somewhat higher activity at longer wavelengths, as compared to that of cells grown completely in inorganic medium. The persistence of the drop suggests that no significant amount of energy can be supplied to photosynthesis from exogenous respiration. (e) When Chlorella was grown in heavy water, the decline[45] in the yield began at 670 mμ (instead of 680 mμ); no enhancement was observed in these cells. An interpretation of this effect must await studies of the absorption spectra of cells grown in D_2O.

B. Chloroplasts from Higher Plants.

Experiments made on the Hill reaction, using quinone[23], NADP[24] or ferricyanide (in the presence of a catalytic quantity of the dye 2, 6 DCPIP[46a]. (figure 2), revealed a long-wave decline ("red drop") similar to that found in photosynthesis of whole cells. An enhancement effect also has been observed, at least with quinone[23] and NADP[24]. (see section V). This lends support to the previously suggested concept that the Hill reaction and photosynthesis have the same photochemical mechanism.

C. Bacteria.

Uptake of $CO_2 + H_2$ was followed in Rhodospirillum rubrum suspensions at certain selected wavelengths, beyond the major peaks of bacteriochlorophyll, i.e. in a region where one would have expected a decline analogous to the "red drop" in photosynthesis. The quantum yield found at 940, 960 and 980 mμ[46b] were all in the range of 0.10, similar to those found at the shorter wavelengths and suggesting absence of a "red drop". Blinks and van Neil[47] noted the absence of an enhancement phenomenon in bacterial photosynthesis.

D. Discussion.

The question "Does the existence of a "red drop" per se prove the presence of two photochemical systems?" must be answered in the negative. If, for a certain reaction, say - the Hill

reaction with a certain oxidant- the long wave form of chlorophyll a (Chl a 680/Chl a 690) were simply inactive, one would still get a "red drop"!

Conversely, the absence of a "red drop" cannot be taken as proof of the absence of two light reactions, because extensive overlapping of the spectral bands of the two systems could make separation of their effects difficult.

However, the presence of both "red drop" and an enhancement effect clearly suggests the existence of two photochemical systems acting cooperatively in the overall process of photosynthesis.

II. "LIGHT CURVES" OF PHOTOSYNTHESIS IN MONOCHROMATIC LIGHT

Studies of the enhancement phenomena are reliable only if the light curves (the plot of the rate of photosynthesis, R, as a function of light intensity, I) are linear in the intensity range of the experiment, so that by combining the two beams, one does not get into the saturating (or at least less sloping) range, suggesting "negative" Emerson effect. Similarly a "positive" effect can be wrongly inferred when one of the light curves is sigmoid in shape (cf. below). This necessitated a careful investigation of the light curves under different conditions.

A. Light Curve with "Knick"

McCloud[48] (as well as Govindjee[8b,49]) had observed that the saturation rates of photosynthesis in various algae were dependent on the wavelength of light; at the longer wavelengths- beyond 680 mμ - an "early" saturation on a relatively low level was reached. These observations were very difficult to interpret, since saturation was supposed to be imposed by the availability of a limiting enzyme, which should be the same whatever the wavelength of light. Because of the narrow range of light intensities used in my earlier experiments, which had led to the above conclusions, we repeated these measurements on Porphyridium over a wide range of intensities. Two wavelengths were selected -- one absorbed primarily by phycoerythrin (546 mμ) and the other by the "long wavelength" form of chlorophyll a (700 mμ). Advantage was taken of the well-known fact that saturation levels are lower at the lower temperatures. Measurements were made at 3-5°C (see figure 3). The 700 mμ light curve seems to approach the same saturation level as that obtained in

green light, in contradiction to the results of McCloud[48] (and my own earlier conclusions[8b,49]). In an independent study, Pickett and Myers[14e] found the same saturation rate at different wavelengths in Chlorella. The cause of the discrepancy between the newer and the earlier results is that the 700 mμ light curve has a peculiar "break", which was mistaken for "saturation", due to the narrow range of intensities studied.

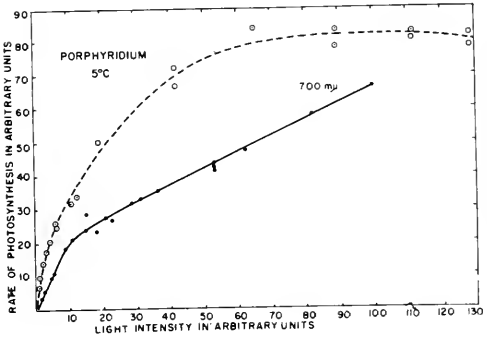

Figure 3. Rate of photosynthesis in Porphyridium as a function of light intensity in two monochromatic beams 545 mμ and 700 mμ measured at 5° C.

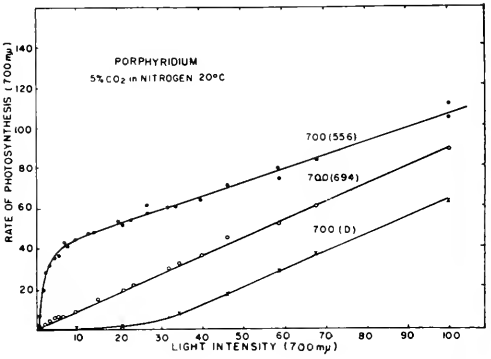

Figure 4. Rates of oxygen evolution, in Porphyridium as a function of light intensity.

My new measurements show a clear "Knick" in the light curve at 700 mμ (figure 3). Kok had observed such broken light curves (see Rabinowitch[50] for a discussion of this "Kok effect"). Hoch and coworkers[20] recently reported light curves of the same type in Anacystis. Jones and Myers[14d], in an independent study, also observed such curves in Anacystis.

B. S-Shaped Light Curves.

Figure 4 (lowest curve) shows a light curve obtained at 700 mμ in Porphyridium cruentum at 20°C under anaerobic conditions. It is clearly S-shaped. The middle curve shows that addition of another beam of the same wavelength, makes the curve linear. With Bannister & Vrooman[17], I believe that under nitrogen the lower segment of the S-shaped "light curve" is due to immediate consumption of the oxygen evolved in photosynthesis; the oxygen production in photosynthesis, is therefore not registered by the polarograph. At higher intensities, oxygen production increasingly exceeds oxygen consumption (thus destroying the external anaerobic condition). (Reference may be made here to a paper by

Franck et al[51] which suggests several other possible interpretations of S-shaped light curves obtained in white light under anaerobic conditions).

S-shaped light curves are well-known in bacterial photosynthesis[50]; recently they have been found also for the production of ATP by chloroplasts.[52] We observed[46a] an S-shaped light curve for oxygen production by spinach chloroplasts. (The reaction mixture contained excess of ferricyanide and traces of 2,6 DCPIP).

A method to observe enhancement in non-linear light curves is through the experiment shown in figure 4. (1) The rate of reaction as a function of light intensity of the far-red light (A) is first measured (lowest curve, figure 4) (2) The same is done in the presence of another beam (B) of far-red light, of the same wavelength (middle curve, figure 4) (3) The same is done in the presence of supplementary beam (C); the intensity of which is adjusted to give the same rate of reaction as that produced by beam (B). Enhancement is then the difference between the upper curve and the middle curve or

$$E = \frac{R_{1,2} - R_1}{(R_{2,2'} - R_2)} \qquad (1)$$

where E = Emerson enhancement, $R_{1,2}$ = rate in both supplementary and far red beams, R_1 = rate in supplementary beam alone, R_2 = rate in far red beam alone, and $R_{2,2'}$ is the rate in combined far-red beams.

C. Light Curves with Continuously Decreasing Slopes (early saturation effects).

Light curves for various Hill reactions, in general[53] show a decreasing slope with increasing light intensity, (i.e. only a very short linear range is available). This raises the question of the validity of the results[18,21] in which no Emerson enhancement was reported for the Hill reaction. The 650 mμ light curve for NADP reduction shows the above mentioned early bending of the light curve.[24b,54] Due to early "saturation" effects, two beams of 650 mμ light, given together, give a rate smaller than the sum of the two separate rates.[54]. True enhancement can be calculated by taking this fact into account by means of the equation:

$$E = \frac{R_{1,2} - (R_{1,1}' - R_1')}{R_2} \qquad (2)$$

where the symbols have the same meaning as in equation (1), except that R_1', is the rate due to the second (supplementary beam) alone, the intensity of the latter being adjusted so as to give the same rate as that of R_2.

III. ENHANCEMENT EFFECT AND THE TWO PHOTOCHEMICAL SYSTEMS

A. Action Spectra.

The action spectra of the enhancement effect, measured with a background of far-red light, led to the identification of pigments present in one of the two postulated photosystems (system II). Of course, these action spectra do not follow the true absorption curves, but rather the"fractional-absorption" curve of the pigments belonging to system II. Similarly, the action spectra obtained in the presence of background light of shorter wavelength, suggest the fractional absorption curve of pigments in system I. From our previous studies[8], we concluded that system II in diatoms and green algae includes the short-wave form of chlorophyll *a* (Chl *a* 670). (See figure 5 for the absorption spectrum[55] of *Chlorella*, showing bands corresponding to the two forms of chlorophyll *a*). Light energy absorbed by chlorophyll *b* is assumed to be transferred to Chl *a* 670. System I is mainly composed of the long-wave forms of chlorophyll *a* (Chl *a* 680/Chl *a* 690 and "Chl *a* 700"). In figure 6, the solid curve refers to the action spectrum of the enhancement effect in the presence of 700 mμ background light, suggesting the identification of chlorophyll *b* and Chl *a* 670 as components of system II. The dashed curve refers to measurements in the presence of 650 mμ background light. The ratio of the rate due to far-red light to that due to supplementary light is kept constant (1:2). Unlike Myers and Graham[14c], we observed enhancement of 650 mμ action by 670 mμ light. It, thus, appears that Chl *a* 670 occurs in both systems. On the other hand, the long-wave chlorophyll *a* forms belong exclusively to system I; while chlorophyll *b* belongs exclusively to system II.

Soret bands. Fork[11] has shown that, in red algae, in the action spectrum of enhancement with a background of green light, system I exhibited a Soret band. This was confirmed by Blinks.[15] The demonstration of this band in green algae and diatoms has not met with success in our hands, perhaps due to a strong overlap of ab-

sorption bands of systems I and II in the blue end of the spectrum.

The action spectrum of the enhancement effect with a 700 mμ background light in Chlorella[8c], showed, however, a hump around 440 mμ, in addition to several other bands, due to chlorophyll b and Chl a 670. This 440 mμ band may be due to the Soret band of Chl a 670.

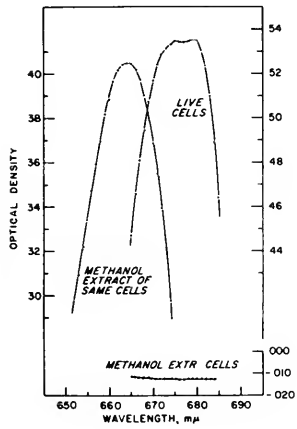

Figure 5. Absorption spectrum of Chlorella and pigments from Chlorella, (measuring band width 1 mμ, after Cederstrand).

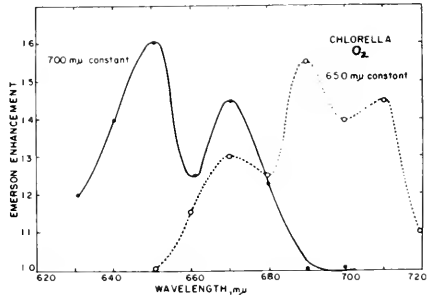

Figure 6. Action spectra of enhancement effect, in Chlorella, as a function of wavelength.

B. LIGHT INTENSITY AND THE ENHANCEMENT EFFECT

Enhancement was measured, both as a function of the intensity

of the supplementary light when 700 mμ light is kept constant, and as a function of increasing intensity of far-red light, when the supplementary light is kept constant. Figures 7 and 8 show that: (1) With an increase in the intensity of supplementary light, the enhancement reaches a plateau and then declines (perhaps we reach the region where the slope of the light curve decreases). (2) With an increase in the intensity of 700 mμ light, the enhancement decreases. Similar results were obtained with 650 and 700 mμ light, and with 670 and 700 mμ light. No enhancement was seen between 690 and 700 mμ at any of the intensities used. Due to the use of manometry in the above measurements, effects of light on the oxygen uptake could not be avoided. With this in mind, we checked part of our results in Chlorella with a mass spectrometer[19]. Enhancements by factors up to 8-10 could be obtained by selecting the appropriate intensity and wavelengths, (650 mμ and 720 mμ). Enhancement increases with an increase in the intensity of 650 mμ light, and decreases with an increase in the intensity of far-red light (at 720 mμ).

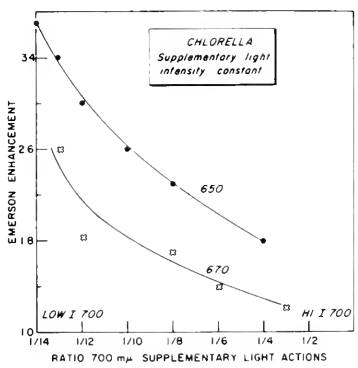

Figure 7. Enhancement effect, in Chlorella, as a function of far-red light intensity.

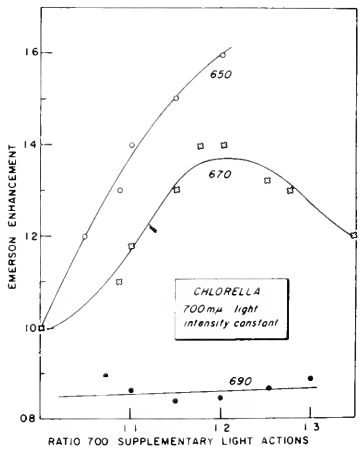

Figure 8. Enhancement effect, in Chlorella, as a function of supplementary light intensity.

IV. ENHANCEMENT EFFECTS IN CHLOROPLAST REACTIONS

We have consistently observed the enhancement phenomenon in the Hill reaction with isolated chloroplasts, using quinone[23] and NADP[24] as oxidants. These experiments clearly show the existence of an enhancement when a far-red beam is supplemented by light of a shorter wavelength. The action spectrum of this effect has peaks at 650 mμ (due to chlorophyll b) and 675 mμ (due to chlorophyll a). (See figures 9 and 10). Enhancement of NADP reduction was accompanied by enhancement of oxygen evolution. The situation may be different with other Hill oxidants, such as certain dyes, or ferricyanide.

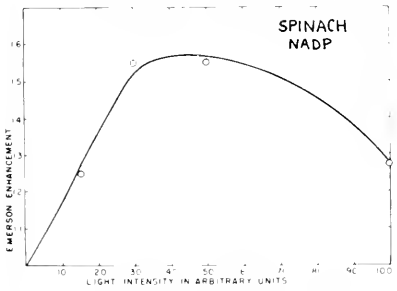

Figure 9 Enhancement in NADP reduction as a function of 650 m light intensity in spinach chloroplasts. (after R. Govindjee and Govindjee and Hoch).

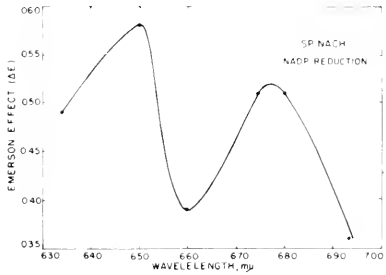

Figure 10 Action spectrum of Emerson enhancement effect in NADP reduction for spinach chloroplasts (after Yang and Govindjee).

V. FLUORESCENCE STUDIES

We reported[32] that the 685 mμ fluorescence excited by 436 mμ light is quenched by the addition of 700 mμ light. This effect can be interpreted in terms of two photochemical systems. Butler[33], Duysens and Sweers[35] obtained similar results. To clarify the mode of action of different oxidants by observing

their effect on the fluorescence yield of "P 700" and Chl a 670, we first measured the action spectra of fluorescence at room, and at liquid nitrogen temperatures.

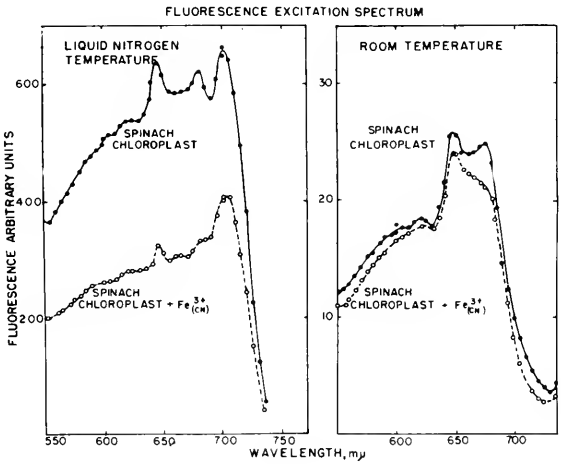

Figure 11. Fluorescence excitation spectra of isolated spinach chloroplasts, measured at 22° C (right) and at -196° C (left); note a twentyfold increase in the fluorescence yield. The appearance of a new band at 700 mμ in the cooled sample is clearly demonstrated; tentatively, it can be assigned to P 700—a pigment (or pigments) absorbing at 700 mμ. Ferricyanide (10^{-3}M) quenches fluorescence yield (see open circles) at all the wavelengths—due to the formation of non-fluorescent oxidation products of the pigments (after Louisa Yang and Govindjee)

First, we show (see figure 11) the action spectrum of fluorescence of a chloroplast suspension, measured at 758 mμ and at -196° C. A peak at 705 mμ appears (which is not present at room temperature). This provides independent confirmation of Butler's[56] prior findings. The peak is believed to be due to P 700.

In Anacystis, fluorescence spectra (see figure 12) (excited by 436 and 605 mμ light, absorbed primarily by chlorophyll a and phycocyanin respectively) show (1) that chlorophyll a fluorescence, when excited via energy transfer from phycocyanin, is much stronger than when it is excited directly; (2) that excitation in the Soret band of chlorophyll a is not transferred to phycocyanin; (3) that energy transfer yield from phycocyanin to chlorophyll a is much less than 100%, as some energy is lost as phycocyanin fluorescence (peak at 650 mμ); (4) upon cooling to -196° C, new peaks appear at 696 mμ, 718 mμ, and a shoulder at 760 mμ.

Govindjee

The ratio of intensities of the 718 and the 685 mμ fluorescence bands at -196° C is higher when chlorophyll a is excited, than when phycocyanin is excited. This suggests two separate chlorophyll a systems: (1) one system in which the energy preferentially goes to the part of chlorophyll a that fluoresces at 718 mμ; and (2) another system in which the energy preferentially goes to the chlorophyll a that fluoresces at 685 mμ.

The 750 mμ peak in the excitation spectrum (see figure 13) is observed consistently, and is perhaps due to P 750 N. The 760 mμ shoulder in the fluorescence spectrum is due to the fluorescence of this pigment. The 696 mμ band may be due to the "unknown trap" of photosystem II (cf. also 57 and 58).

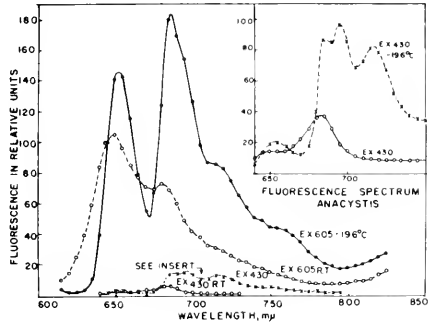

Figure 12. Fluorescence emission spectrum of Anacystis. (after Spencer and Govindjee).

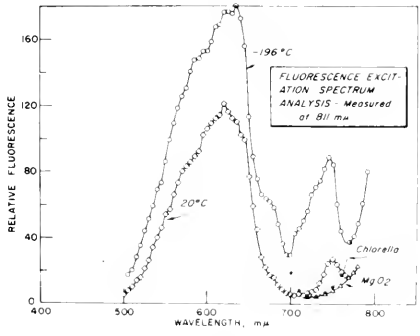

Figure 13. Fluorescence excitation spectrum of Anacystis at two temperatures (after Spencer and Govindjee).

VI. CONCLUDING REMARKS AND SUMMARY

1. The quantum yield of photosynthesis in Chlorella begins to decrease at 690 mμ and declines to about 50% at 730 mμ (at 10° C). No analogous decline was noticed in bacterial photosynthesis. This may mean that a photochemical system of type II is absent in bacteria.

2. A prior knowledge of the shape of the "light curve" (the plot of the rate of the O_2 evolution as a function of light intensity) is necessary to calculate the enhancement factor. A study of this relationship shows the existence of: (a) S-shaped curves, e.g. in photosynthesis under anaerobic conditions, and in O_2 evolution in chloroplasts in the presence of ferricyanide with catalytic amounts of DCPIP; (b) curves with a discontinuity or "knick" (700 mμ light curve for oxygen evolution in Porphyridium,) (c) "light curves" with a rapidly decreasing slope with increasing light intensity.

3. The Emerson enhancement effect first increases linearly with an increase in supplementary light intensity, then saturates and finally declines. It decreases with an increase in the intensity of far-red light.

4. The data presented substantiate the hypothesis of two light reactions involved in both photosynthesis and the Hill reaction. From the two action spectra of the enhancement effect, measured with constant far-red and with constant supplementary beam, respectively, and from fluorescence studies, there emerges a picture of two photochemical systems: System I. This is composed of Chl a 680/690 and its "trap," P 700. The P 700 is nonfluorescent at room temperature, and fluorescent at liquid nitrogen temperature. Its fluorescence peak is at 718 mμ. Some Chl a 670 also belongs to this system. System II. This is composed of Chl a 670, and the "accessory pigments." Chl a is fluorescent at room temperature.

5. In Anacystis, measurements, at -196° C, of fluorescence spectra, excited at 436 mμ (chlorophyll a) and 605 mμ (phycocyanin), show that the relative heights of fluorescence bands at 687 mμ and at 718 mμ are very different. The ratio of the maxima of the 718 mμ fluorescence band to that of the 687 mμ band is greater when Anacystis is excited by 436 mμ light, than when it is excited by 605 mμ light. This suggests the existence of two kinds of chlorophyll a: one that receives its energy primarily from the phycobilins, and another that receives its energy directly from chlorophyll a.

VII. ACKNOWLEDGEMENTS

Continued support from the National Science Foundation (G 19437 and GB 1610) is acknowledged. A grant from the Charles F. Kettering Foundation to Professor Rabinowitch, provided some of the instruments used in the present study. Thanks are due to Maarib Bakri, Carl Cederstrand, Rajni Govindjee, Mary Osbakken, Jobie Spencer and Louisa Yang for their valuable help in this investigation. Part of the work presented here was done at RIAS, Baltimore, and at the Carnegie Institution of Washington, Stanford. My thanks are due to Drs. Eugene Rabinowitch, Bessel Kok, George Hoch, Olga Owens, C. Stacey French, David Fork and N. R. Murty for their interest.

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SEPARATION OF THE EFFECTS OF TWO PHOTOCHEMICAL REACTIONS BY STUDIES OF OXYGEN EXCHANGE

C. S. French

SUMMARY

In the red alga Porphyridium red light, absorbed by chlorophyll a, produces some substance that is an intermediate in the process of O_2 evolution. This material is used up by green light which is absorbed by phycocyanin while O_2 is produced. Its presence after red illumination may be detected either by the enhanced photosynthesis from a short exposure to green light or by the increased rate of O_2 uptake which it causes. No evidence could be found for the post-illumination survival of a comparable green light product. Some other investigations of photosynthesis in flashing monochromatic light are described.

INTRODUCTION

By recording rates of O_2 evolution polarographically[1,2,3] it is possible to see some of the consequences of interactions[4,5] between the two photochemical processes in whole cells or in chloroplasts. Perhaps the most striking evidence for a complex interlocking of photochemical and dark reactions is in the time-course curves for O_2 evolution during the induction period of photosynthesis. These curves have an extraordinary range of variation, yet they are obviously composed of underlying basic characteristics--an initial sharp spike, a later rounded spike, then a dip and a slow rise, perhaps followed by a slow decline. Various influences--wavelength of light, temperature, added chemicals, hydrostatic pressure, and previous treatment of the material, all produce systematic changes of the specific features of the rate curves during the induction period.

Learning to read the meaning of these curves in terms of two or more photochemical reactions acting on limited pool sizes of intermediate substances is still a distant objective. Intuition backed up by the application of mathematical expressions for rates of interrelated chemical reactions of a hypothetical nature[4] is only a beginning. Eventually the biochemical transformations and the kinetic observation on rates of O_2 evolution must be explainable in the same terms.

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THE POST-ILLUMINATION EXCHANGE OF O_2

Brackett, Olsen, and Crickard[6] found a stimulation of respiration in Chlorella immediately after illumination, and Blinks[7] saw the effect using two beams of light.

The difference between green and red light in producing an increased uptake of O_2 after the exposure is shown in figure 1. The

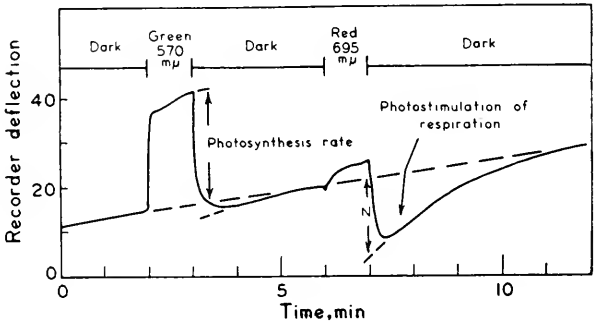

Fig. 1. The time course of photosynthesis in red and in green light for Porphyridium. Red light makes a product that increases the rate of O_2 uptake during and after exposure. French and Fork[5].

drop in rate of O_2 evolution after a light exposure may take over a minute to become complete even in the presence of a large respiratory stimulation induced by light. After the rate has reached its minimum value it climbs back to the original dark level as the light-induced material that gives increased O_2 uptake is consumed. Its half-life in Porphyridium is about 1.1 min.

Of course it is possible to explain the observed curves by assuming two opposite effects of light on O_2 exchange. From this point of view we would have an inhibitory effect of light on O_2 uptake that decayed fairly rapidly, superimposed on a more persistent stimulation of O_2 uptake. The alternative explanation for the slowness of the return of the rate to its pre-illumination level is that O_2 production actually continues at a more or less exponentially decreasing rate after the light is turned off. This continuing O_2 evolution has its counterpart in CO_2 uptake following a light exposure--the "pickup" experiments of McAlister[8]. The post-illumination extension of O_2 production[9] is better explained by the idea of parallel rather than consecutive photochemical reactions. According to this concept of Brody and Brody[10]

and of French and Fork[4,5], reviewed by Clayton[11], O_2 comes off from a reaction involving both photoproducts. Following an exposure to a wavelength previously activating one of the pigment systems, the lingering O_2 evolution decreases slowly. However if the other photoproduct is continuously produced by exposure to a background light which activates the other pigment system the decrease in rate is much more rapid, as seen in figure 2. Thus by speeding

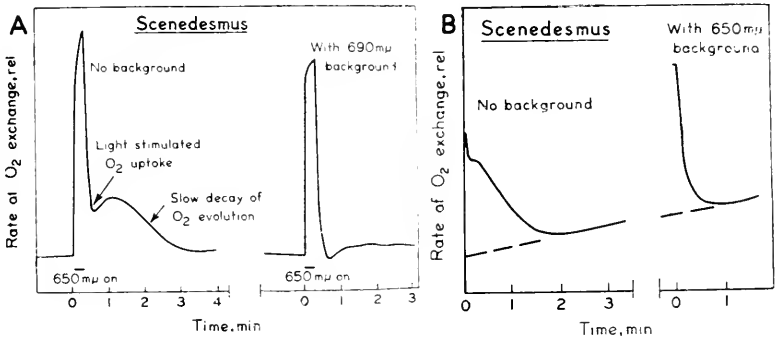

Fig. 2. (A) The rate of O_2 exchange from a 20-second exposure at 650 mμ in Scenedesmus. A background light of 690 mμ increases the rate of decay of the lingering O_2 evolution so much that the opposing light-stimulated O_2 uptake becomes evident.

(B) The decline in rate of O_2 evolution in Scenedesmus after a 2-minute exposure to 700 mμ. With 650-mμ background the decline is much steeper. French[9]

up the utilization of the O_2-evolving products this reaction can be completed quickly. When this is done with a background beam the light-stimulated uptake of O_2 shows more clearly in the post-illumination part of the time-course curve, as is also evident in figures 1 and 2.

Because the increased rate of O_2 uptake at very low light intensity starts before photosynthesis does, its magnitude for different wavelengths can be measured by the small negative deflection at the start of a light exposure. The action spectrum for respiratory stimulation was measured by plotting the initial negative spike and also by the magnitude of the increased O_2 uptake following an exposure. The spectra of figure 3 show that the increased O_2 uptake is induced by light absorbed by chlorophyll while, as has long been known[2,12,13], O_2 evolution is far greater in light absorbed by the accessory pigment system. An interesting characteristic of these spectra is that they show very little if any evidence for the participation of phycoerythrin in the

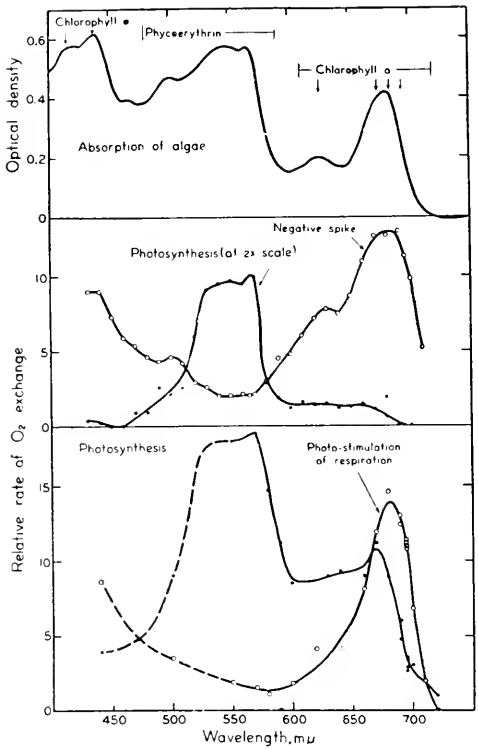

Fig. 3. The action spectra for 0_2 production in Porphyridium compared with that for production of the substance that increases the rate of 0_2 uptake measured in two different ways. The top curve is the absorption spectrum. French and Fork[5].

chlorophyll system found by Duysens and Amesz[14] measuring cytochrome changes.

THE LIFETIMES OF THE PRODUCTS OF THE TWO REACTIONS IN THE DARK

Myers[15] found that the Emerson enhancement effect could be seen if the two beams were rapidly alternated as well as when they were given together. This implies that at least one of the photochemical reactions makes a product that survives the exposure producing it and that this product makes possible a more efficient

utilization of light in the other beam. However, this experiment does not tell which beam makes the enhancing product.

Experiments[9] with light exposures of a few seconds duration have settled this question, at least for Porphyridium. They showed that more oxygen was produced by a green exposure if it was preceded by a red exposure (Table 1). The converse effect: an

TABLE 1. Peak Rates from 3-sec Light Exposures in Porphyridium

Green alone	19.4	
Green, 20 sec after red	23.0	Enhancement
Red alone	17.1	
Red, 20 sec after green	14.0	Inhibition

increase in yield in red light following green was not seen.

In these experiments on Porphyridium I did not see any increase during the dark time between the exposures as was found in both Ankistrodesmus and in chloroplasts by Whittingham and Bishop[16]. In Porphyridium the amount of enhancement merely declined during the dark time with a half-life of about 18 sec.

Recently I looked for possible products of green light action that might enhance photosynthesis by red light, but that might have such a short survival time in the cells as to have escaped detection with exposures of a few seconds and with corresponding dark intervals of 20 seconds. In these experiments 10 ms paired flashes of red and of green were given 50 ms apart but with long dark times of 500 ms between successive exposures to the two flashes. Even the long dark times were so short that the decay of the red light product was insignificant. No difference was found if the order of the flashes within each pair was reversed. Red followed by green gave the same rate as did green followed by red. Enhancement in Porphyridium therefore seems to be due to a red light product that is used by green light. This finding implies that the common method for calculation of enhancement as an increase in photosynthesis in red light by added green should be reconsidered. At least in Porphyridium the utilization of a red light product by green light given later shows that the enhancement could perhaps better be expressed as an increase in the rate of photosynthesis by green light when red is given before or during the green exposure.

C. S. French

THE RATES OF UTILIZATION OF THE PHOTOPRODUCTS DURING ILLUMINATION

The flashing light experiments of Emerson and Arnold[17], later followed up with improved methods by Tamiya[18], Kok[19], and others, were done before it was realized that more than one photochemical reaction was involved. The reduction in the yield per flash as the dark period was shortened was interpreted as measuring the time-course of a dark reaction initiated by the photochemical act. The time-courses for the dark reactions following each photochemical reaction are certainly different, as we have seen with the long flash experiments. The basic idea was to follow the time-course for the utilization of the products of each photochemical reaction as the intensity of the background light absorbed by the other pigment system is varied. It has not yet been possible to find conditions giving flash saturation over a range of dark times for one light color in the presence of a continuous background light activating the other pigment system. The range between the rate at flash saturation for one color with background light of another color and the ceiling imposed by the saturation level in continuous light leaves very little scope for significant measurements of the yield per flash with differing dark times.

Some results with flashing light will be mentioned: In these experiments with Porphyridium at 5° C nearly identical saturation rates with continuous light were found in the two colors. This result differs from those of McLeod[20] with other species at room temperature where the rate at saturation was found to vary with wavelength.

The flash-saturation level at 5° C is identical with red and with green light. With flashes of 9 ms at 95 ms dark intervals the flash-saturation rate in both red and green light was about 60 per cent of the rate at saturation with continuous light. With 1.5 ms flashes and about the same dark time the available intensity did not produce flash-saturation but at least with red light at several intensities the rate appeared to extrapolate to about the same value found for the longer flashes. However with red flashes of 1.5 ms and dark times of 535 ms a flash-saturation level of about 21 per cent of the continuous saturation level was approached. These flashes were also given with a continuous green background light that produced a rate 25 per cent of saturation. A small enhancement of the rate was seen but flash saturation was not attainable. Evidently greater familiarity with the response of Porphyridium to various regimes of flashing monochromatic light will be necessary before the appropriate experiments leading to the above-outlined objectives can be planned. Various exploratory experiments have, however, turned up some unexpected effects described in the next section.

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SOME EFFECTS OF MONOCHROMATIC FLASHING LIGHT IN PORPHYRIDIUM

Further effects of flashing light selected in wavelength to activate preferentially one or the other pigment system were seen. A striking difference between green and red was found in the variation of photosynthetic rate as the speed of the sector was varied. This kind of a flashing light experiment, in which both the length of the flash and the dark time vary together, has an advantage over the independent variation of either, in that the total amount of light given the cells per minute remains constant. Measured changes in the steady-state rate are far less susceptible to errors from unexpected causes than are calculations of yield per flash from widely differing rates. The interpretation of the results is, however, not as straightforward as for experiments in which only the dark time was changed. Figure 4 shows such an

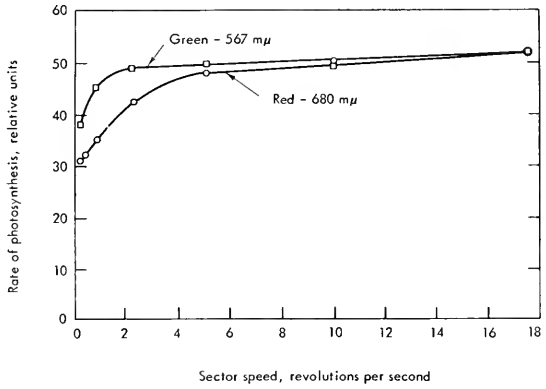

Fig. 4. The variation of photosynthetic rate of Porphyridium with sector speed in two colors of flashing light. Conditions: artificial sea water, 5 per cent CO_2 in air, about 23° C, sector opening about 1.2 per cent of circumference.

experiment in which the intensities were adjusted to give the same rate in green and in red at the highest sector speed. With red light the rate was more strongly influenced by the distribution of the light in time than it was with green light.

At low light intensity the differences between the rates with high and low sector speeds are large. However, at higher intensity the differences become smaller as is evident from figure 5.

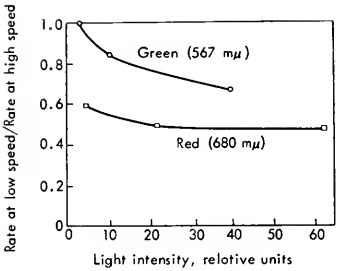

Fig. 5. The decrease in photosynthetic rate at low sector speed at different intensities of red and green light.

A particularly interesting difference in the response to single flashes of 50 ms given at 21.5 sec intervals is illustrated in figure 6. Here we see two different manifestations of the enhancement effect. The top curve shows green flashes given with

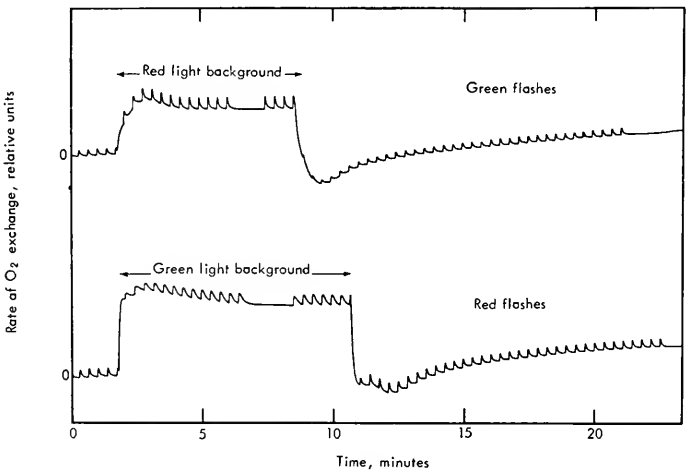

Fig. 6. The rate of oxygen exchange following 50 ms flashes given repetitively with and without complementary background light.

and without a continuous red background. The spike height, that is, the initial rate of 0_2 evolution, is increased by the continuous red light. The time during which 0_2 is produced after the

flash, however, remains the same with and without the red background.

On the other hand, the red flashes behave differently. The height of the spikes from red flashes does not increase when a green continuous background is given. In this case the enhancement shows up as a prolongation of the time of O_2 evolution following the flash. For flashes of either color more O_2 is produced per flash when the other pigment system is illuminated by continuous light, but the kinetic nature of the increase contrasts sharply. Similar experiments were carried out last summer by the Govindjees in far more detail.

So far only a beginning has been made in developing a kinetic picture of the relations between the two light reactions of photosynthesis with their associated dark reactions, as measured by O_2 evolution.

COMMENTS ON ABSORPTION AND ACTION SPECTRA WITH PEAKS NEAR 700 mμ

One of the current basic questions is whether or not all action spectra giving peaks near 700 mμ can be explained on the basis of an activity of C_a 685 which is driven in the opposite direction by the overlapping absorption of C_b 650 and C_a 670. If not, there must be three rather than two separate photochemical reactions in the photosynthetic process. The work of the Witt group[21] has lead them to this conclusion.

Near 700 mμ and beyond, the absorption of C_a 685 greatly predominates over the absorption by the tails of the accessory pigment system components C_a 650 and C_a 670. Effects due to C_a 685 absorption which are reversed by action of the accessory pigment system should therefore give an apparent action spectrum peaking at or beyond 700 mμ.

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CHROMATIC TRANSIENTS AND ENHANCEMENT RECORDED BY THE GLASS ELECTRODE

L. R. Blinks

Blinks and Skow [1] showed that a glass electrode surface in direct contact with tissues or cell suspensions could be used to indicate the exchange of CO_2 (or other acids) during respiration and photosynthesis. Transients were recorded, with "acid gushes" sometimes occurring during the first moments of illumination, preceding the expected rise of pH. These were later detected manometrically by Emerson and Lewis [2] and identified as CO_2 gushes. They have since been designated the "First Emerson Effect." The cause of this acid gush is still unknown, and deserves further study.

Later applications of the glass electrode were made by Gaffron [3] Rosenberg [4], and most recently by Blinks and van Niel [5], who followed the consumption of acetate in Rhodospirillum rubrum by its means. They found no evidence of photosynthetic enhancement in these bacteria, when the latter were simultaneously illuminated at two or more critical absorptive regions of the constituent pigments. There was only a simple addition of rates. While this was in accord with expectation, since there is no oxygen evolving mechanism in R. rubrum, it nevertheless seemed desirable to employ the same method with algae, where both manometric and polarographic oxygen evolution measurements had shown good enhancement [6,7,8].

In the present paper both enhancement and chromatic transients are shown in the photosynthetic fixation of CO_2 by several algae, when illuminated simultaneously or consecutively with essentially monochromatic light absorbed by chlorophyll a and one or another of the "accessory pigments" such as chlorophyll b, phycobilins or carotenoids.

Two arrangements were employed: one, best adapted to suspensions of cells or filaments in fluids, gives a progressive, cumulative record of the CO_2 exchange; the other, best adapted to thin tissues or membranes yields steady state, direct rate

measurement (as does the platinum polarographic electrode in contact with the tissue). The former, fluid type may be simply a conventional glass electrode, plunged into the cell suspension [3]. If only small amounts of material are available, or one wishes to use a narrow beam of light (as from a monochromator slit), a bulb of 015 Corning glass may be used to contain the suspension. This may be spherical or elongated into a cylinder as in the "everted" glass electrode employed by Blinks and van Niel [5]. The bulb, filled with suspension, is immersed in an external buffer, with the pH changes now being recorded in the opposite sense to the normal, immersed electrode. Connections are made by calomel electrodes to the recording system (a Beckman Type G meter and a "Speedomax" or "Servo-riter" recorder.)

For thin thalli or membranous algae this system is not as well adapted as the "film technique." Here the thallus is held in direct contact with the glass surface, either with a band of cellophane, or in the ultimate modification, by capillarity. The former arrangement is immersed in sea water, exactly as with the polarographic platinum electrode. But this tended to give slow responses; even when the volume of sea water could be kept small, CO_2 equilibrated slowly with sea water by diffusion or convection. Flow or stirring improved this situation (as with the oxygen electrode). But, finally, with certain algae, the arrangement shown in Fig. 1 was utilized. Some intertidal algae such as Ulva, Enteromorpha or Porphyra are often exposed for many hours to the moist air of the littoral zone, without obvious damage or decrease of photosynthetic rate. Consequently it was decided to try the alga in moist air only against the glass electrode. The latter was largely coated with a hard wax ("Ceresin") except for a "window" of about 3 mm. x 25 mm. Over this glass surface was placed a moist, fresh thallus of Porphyra or Ulva, and the electrode then mounted in a closed jar containing a little sea water at the bottom. The latter made contact with a tab of the tissue which projected beyond the electrode; this tissue, with resistance so much less than that of the glass electrode, made essentially equal contact with all parts of the exposed electrode surface. The fluid, consequently the total buffering to be altered by CO_2 exchange, was now reduced to that in the tissue itself, plus the thin adherent films of sea water on its inner and outer surface. As expected, the pH changes were now much more rapid; also, the pH now reached a new steady state determined by the photosynthetic rate, and the diffusion of gaseous CO_2 from the atmosphere.

L. R. Blinks

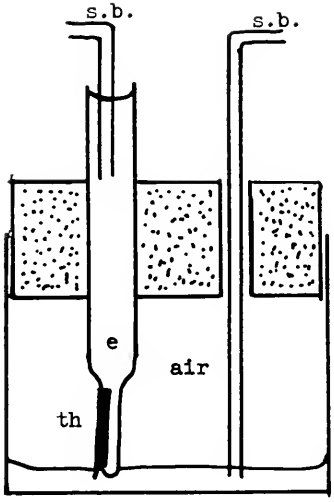

Figure 1. Apparatus for measuring pH changes due to CO_2 exchange in membranous algae. A thallus (th) is held by capillarity against the active glass electrode surface (the remainder being waxed). The tip of the tissue dips into sea water. KCl-agar salt bridges (sb) connect to calomel electrodes.

At the beginning of the recent work on algae, a few records were taken with the "fluid" arrangement, in an everted electrode especially with the filamentous green alga Chaetomorpha, and the delicate branched red alga Antithamnion (in normal sea water). The results with the former are shown in Fig. 2. The pH drifts downward in the dark, then after a slight "acid gush" increases rapidly in the light. With less intense monochromatic light at 702 mμ, compensation was just reached, at which the pH remained constant. The same was approximately true at 645 mμ (absorbed by chlorophyll b). If the intensity of either of these was doubled, the rise of pH just about equalled the fall in the dark. On the other hand if the two wave lengths were given together, the rise of pH was about twice as fast as the previous fall in the dark. This gives an enhancement of some 50% or a factor E of 1.5, where E= a,b (simult.)/ a + b. Just as with oxygen evolution, CO_2 fixation shows good enhancement.

With the second arrangement, (membranous algae) normal air (saturated with water vapor) was passed slowly through the jar; in a few cases air enriched with 1% CO_2 was used, but this tended to buffer the tissue too well, and gave smaller pH changes on illumination. The tissues, especially Enteromorpha and Porphyra perforata, tolerated this exposure to moist air very well, and could be used for several days. In the case of Porphyra, some of the intercellular wall materials (comparable to agar) no doubt

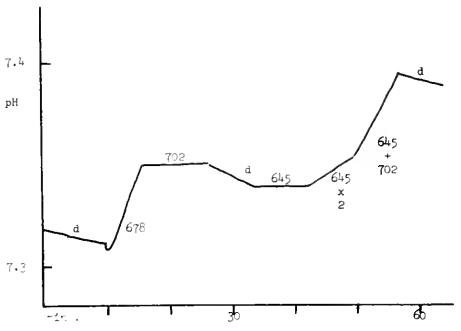

Figure 2. pH changes of sea water produced by a green filamentous alga, Chaetomorpha aerea, contained in an "everted" glass electrode. Respiration (d) caused a slow fall of pH: red light caused a small "acid gush," followed by a rapid rise of pH. Far red light of the same intensity produced compensation, with no pH drift, as did 645 mμ. Doubling the intensity of the latter doubled this rate, while 645 + 702 mμ show good enhancement, with about 50% increase of rate over the expected addition of the individual wave lengths.

contributed to the buffering of the system, since they are capable of cation exchange, including H-ion as shown by Eppley and Blinks (9), and Eppley (10).

Fig. 3 shows the change of pH produced by illumination with red (678 mμ) and green (566 mμ) light respectively in the case of Porphyra Nereocystis. The former produces a rapid pH rise, followed, in the course of about 5 minutes, by a slow decrease. The green light causes a slower rise, sometimes with an induction period (rise, fall and later rise). Darkening causes prompt fall of pH after red light, but often a slower one after green light.

Fig. 3 also shows chromatic transients, produced by alternation of red and green light. As might be expected from the light-dark time course the red transient consists of a cusp (a) followed by a slow fall to a steady state; the green transient of a rapid drop, followed by rather quick recovery. The size of these chromatic transients was usually 10 to 15% of the steady rate, but occasionally, especially with tissue which had been on the electrode for considerable time, might become much larger, reaching 50% in extreme cases. It is believed that some of these represented warming of somewhat dehydrated tissue. In any case it was greatest in the case of a 678 mμ interference filter which had an infra-red transmission band as well.

L. R. Blinks

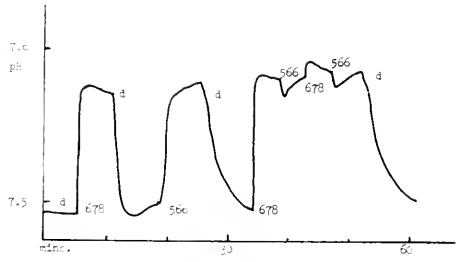

Figure 3. pH changes with the "film" or membrane technique, using Porphyra Nereocystis, a red alga, in direct contact with electrode as in Fig. 1. The pH, nearly stationary in the dark, rises rapidly when the tissue is illuminated at the chlorophyll a peak (678 mμ); there is also a rapid fall in the dark (d) with a slight overshoot (probably due to increased respiration). Green light, at the absorption peak of phyco-erythrin (566 mμ) caused a slower rise (with initial induction), and was followed by a slower fall in the dark. The 2 wave lengths were next alternated, giving chromatic transients with magnitude some 15% of the photosynthetic steady rate.

Rapid alternation of the two wave lengths did not cause any appreciable rise of the steady state rate; this is not in agreement with the "flicker effect" in oxygen evolution (6).

At atmospheric CO_2 content, evidence of an initial acid gush was seldom found on these records. But this is to be expected, since Emerson (2) showed the CO_2 gush to be more prominent at higher CO_2 tensions.

The Second Emerson Effect (enhancement) is well shown by the glass electrode. Fig. 4 indicates the extent of this in P. Nereocystis. The rate (E=1.5) with two lights is at least 50% higher than that predicted from simple addition of the two monochromatic rates. The transients following added red light (with tissue now exposed to green light only), become very conspicuous, the pH dropping almost to the dark base line before recovery sets in.

Lesser transients and enhancement of the CO_2 exchange were found between chlorophyll a (at 678 mμ) and phycocyanin (at 615 mμ), probably because of a greater overlap in absorption by the latter compared to phyco-erythrin. Red algae also showed CO_2 transients and enhancement between phyco-erythrin and the blue absorption region of chlorophyll a. No evidence of carotenoid participation in either photosynthesis or transients was found, however.

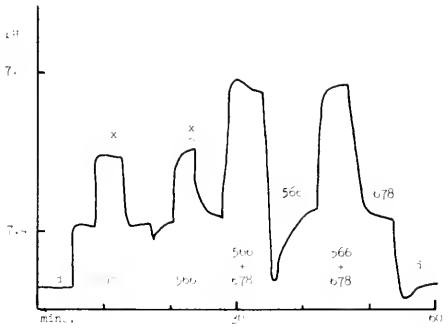

Figure 4. Photosynthetic enhancement in Porphyra Nereocystis. Exposures to red light (678 mμ) and green (566 mμ) were adjusted to give essentially equal responses. Doubling the intensity of each light doubled the response. Adding red to green light (566 + 678) however, produced some 50% greater rise of pH, with an enhancement factor (E) of about 1.5. Note the very profound undershoot, almost reaching the respiration base line, when red was turned off. There is conversely a rather slow fall of pH on darkening after green light.

In green algae, good chromatic transients and enhancements between chlorophylls a and b were obtained by the glass electrode. Brown algae have not yet been tested by this method, most of them having thalli too thick for rapid gas equilibration, hence giving slow responses.

Perhaps the greatest surprise in the CO_2 transients is the direction of the cusps due to chlorophyll and the accessory pigments. Oxygen evolution is initially much higher when the accessory pigments are absorbing (though followed by a pronounced depression); however, CO_2 fixation starts more slowly during accessory pigment absorption and then rises. Conversely, absorption by chlorophyll causes a slower rise of oxygen evolution, whereas CO_2 consumption starts off quickly and falls away with time.

These differences are no doubt to be related to the different rates of TPNH and ATP formation by the chlorophyll and accessory pigments; but also to the ribulose and other pool sizes in the carbon fixation cycle. Until these biochemical factors (and their rates) are related to individual pigment participation, external measurements of such raw materials or products as carbon dioxide and oxygen can only suggest, but certainly not answer, the kinetic problems which must be involved.

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OBSERVATIONS ON THE FUNCTION OF CHLOROPHYLL *a* AND ACCESSORY PIGMENTS IN PHOTOSYNTHESIS

David C. Fork

SUMMARY

The "inactive" chlorophyll of red algae in red or in blue light was seen when action spectra for photosynthesis were determined at low temperatures but was abolished when action spectra were determined with green background light (absorbed by phycobilins). Enhancement was thus not limited to the red absorption band of chlorophyll *a* but occurred as well in the blue.

Enhancement spectra are given for green, brown, and red algae. The results are considered in terms of the Emerson enhancement effect that efficient photosynthesis requires dual excitation of accessory pigments and long-wavelength chlorophyll.

Two different light reactions, each sensitized by a different pigment system were seen in chloroplasts lacking added Hill oxidants. An action spectrum for the O_2-production spike had a peak at 669 mμ, a far-red limit around 713 mμ, and suggested the functioning of the accessory or short-wavelength chlorophyll system, while the action spectrum for the regeneration of the O_2 spike by light had a peak near 700 mμ which indicated the participation of the long-wavelength chlorophyll system.

INTRODUCTION

Until the discovery by Emerson[1] of what has become known as the enhancement effect of photosynthesis the so-called accessory pigments were considered to function only insofar as they were able to pass along their absorbed light energy to chlorophyll *a*.

Red algae presented an apparent exception since Haxo and Blinks [2] found that photosynthesis was efficiently sensitized by phycobilin-absorbed light. However, absorption of light by chlorophyll *a* itself resulted in only feeble O_2 production. Since fluorescence studies by Duysens[3] and French and Young[4] indicated that

chlorophyll a was the ultimate acceptor of phycobilin-absorbed light, the idea arose that a certain fraction of red algal chlorophyll a must be photosynthetically inactive. In a study of inactivity of chlorophyll in the far red (the so-called far-red decline) Emerson and co-workers[5,6] found that lowered temperatures extended the efficiency of photosynthesis to longer wavelengths, both in Chlorella and in the red alga Porphyridium cruentum.

ACTION SPECTRA FOR PHOTOSYNTHESIS OF RED ALGAE AT LOW TEMPERATURES

Since the action spectra determined by Haxo and Blinks[2] for red algae were done at room temperature it was suggested that similar measurements at lower temperatures might reveal a more active chlorophyll. Action spectra given in figure 1 for red algae measured at 1° and 5° and again at 20 and 27° C were almost identical

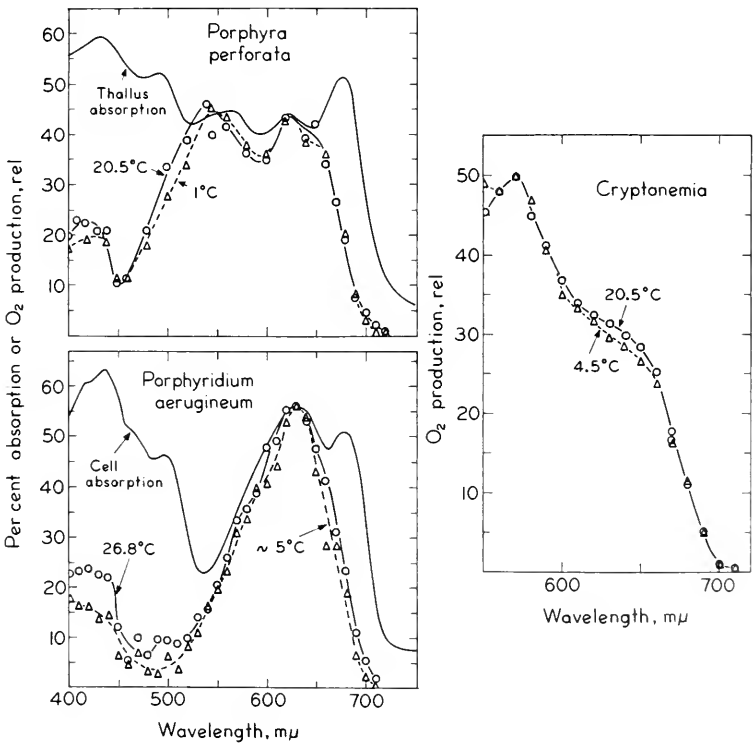

Fig. 1. Action spectra for 0_2 evolution in red algae determined at different temperatures. Fork[7].

in shape and indicate that a temperature effect alone is not responsible for the low photosynthetic activity of these algae in red and blue light.

THE EMERSON ENHANCEMENT EFFECT IN RED, BROWN, AND GREEN ALGAE

Emerson and co-workers[6] observed that the quantum yield of Chlorella could be improved in longer wavelengths if supplementary light was provided during the measurements. Upon closer examination of the wavelength dependence of this enhancement effect, Emerson[1] showed in Chlorella that supplementary light most effectively absorbed by chlorophyll b brought about the greatest increase in yield from a band of far-red light. In Porphyridium the most effective supplementary light was that absorbed by phycobilins.

The Emerson enhancement effect with background illumination of appropriate wavelength can be seen clearly in the action spectra for O_2 production of the red alga Porphyra perforata (figure 2).

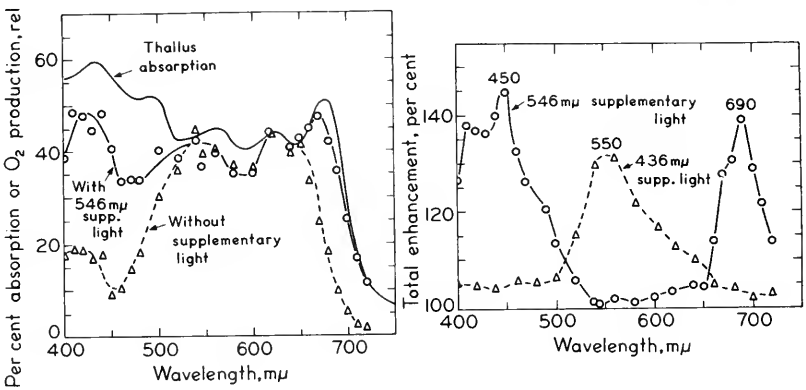

Fig. 2. (left) Action spectra for O_2 production in Porphyra perforata determined at 20.5°C with and without supplementary green (546 mμ) background light.
(right) Enhancement spectra for P. perforata obtained with green (546 mμ) background and blue (436 mμ) background light. Fork[7].

The action spectrum determined without background illumination is similar to that reported by Haxo and Blinks for this species[2]. The action spectrum determined in background light of 546 mμ (strongly absorbed by phycobilins) no longer shows the "inactive" chlorophyll so typical in action spectra of red algae. Green background light sustains enhanced photosynthesis in both red and

the blue regions of chlorophyll a absorption. Blinks[8] has also observed enhancement with combined blue and green wavelengths. Enhancement, expressed as the ratio of the photosynthetic response obtained when two beams are presented simultaneously divided by the sum of their separate responses, is also given in figure 2 for Porphyra in green and blue background light. Cryptopleura crispa, another intertidal red alga, showed a response (figure 3) similar

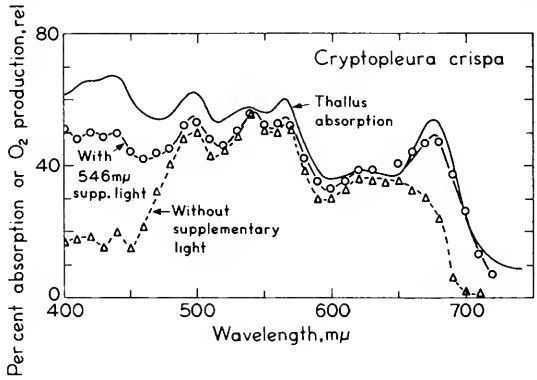

Fig. 3. Action spectra for O_2 production in Cryptopleura crispa (15.5°C) with and without green (546 mμ) background light. Fork[7].

to Porphyra when action spectra were determined without and with green supplementary illumination absorbed by phycobilins. Drouetia rotata, a red alga growing exclusively in deep water where red wavelengths are largely filtered out, was obtained at a depth of 37 m. The thallus of this plant is too thick for ideal use with polarographic techniques; nevertheless, it showed improved chlorophyll activity when simultaneous phycobilin and chlorophyll excitation was provided (figure 4).

These action spectra for red algae demonstrate that the fundamental basis for the enhancement effect is not the pairing of low energy quanta absorbed by chlorophyll a with higher energy quanta absorbed by accessory pigments (or chlorophyll a itself), but rather the requirement for dual excitation of accessory pigments and chlorophyll a for efficient photosynthesis. Red and blue absorption by these algae is similar in that chlorophyll a excitation is effected without appreciable phycobilin excitation. The "inactive" chlorophyll of red algae could better be termed "unenhanced" chlorophyll.

Enhancement spectra for a green and a brown marine alga, Ulva and Endarachne respectively, are given in figure 5. Ulva showed peaks of effectiveness around 485 and 645 mμ corresponding most closely to the absorption by chlorophyll b, while Endarachne

showed peaks at 470 (chlorophyll c) and at 540 mμ (fucoxanthin) and minor peaks at 610 and 660 mμ.

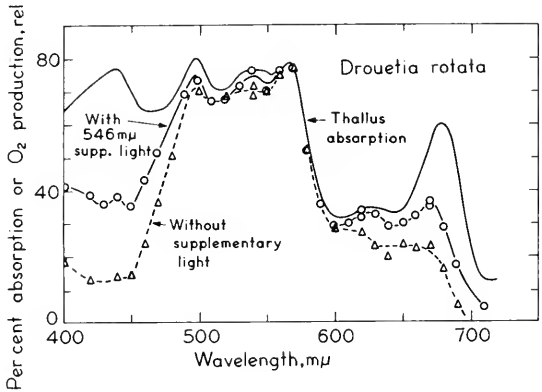

Fig. 4. Action spectra for 0_2 production in Drouetia rotata (10°C) with and without green (546 mμ) background light. Fork(7).

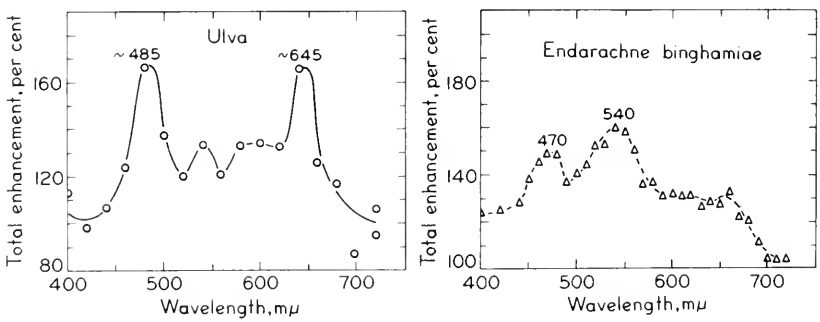

Fig. 5. Action spectra for enhancement in Ulva and Endarachne binghamiae with a background light of wavelengths longer than 680 mμ. Fork(7).

EVIDENCE FOR TWO LIGHT REACTIONS IN CHLOROPLASTS

If a two-pigment mechanism functions in photosynthesis, one would expect to be able to observe the enhancement effect in

chloroplast preparations. The effect can usually be detected without undue difficulty in intact cells; however, it has variously been reported to be present[9] or absent[10] in chloroplast preparations. Govindjee (this symposium) has outlined possible explanations for failure to observe enhancement in chloroplasts.

Evidence for the operation of two light reactions in chloroplasts has come from studies on absorption changes[11], biochemical studies[12,13] and measurements of enhancement[9,14].

The participation of two light reactions in the evolution of O_2 by chloroplasts can be seen clearly in chloroplasts maintained in buffered sucrose or NaCl solutions. While such a system may be considered undefined, it may also be considered unchanged by the addition of substances known to exert a profound effect on the photosynthetic reactions of chloroplasts. Considerable insight into the photosynthetic mechanism may be gained from a study of chloroplasts lacking an added Hill oxidant since their limited capacity for O_2 evolution can be increased by pre-illumination or by a period of darkness.

Figure 6 shows time courses for O_2 evolution upon illumination of chloroplasts in the absence of an added Hill oxidant under anaerobic conditions. The time-course curve for O_2 production shows

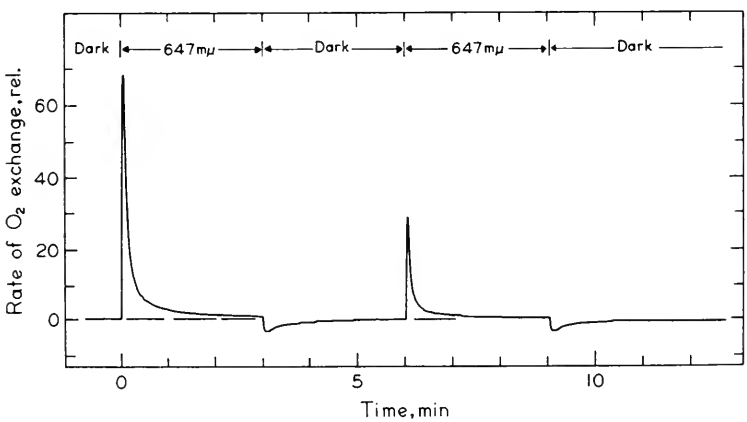

Fig. 6. Time-course curves of O_2 evolution upon exposure of whole Swiss chard chloroplasts to 647 mμ light (1500 ergs $cm^{-2}sec^{-1}$). The chloroplasts, held on a Teflon-covered Pt electrode with dialysis membrane, were equilibrated with 0.4 M Sucrose, 0.05 M K_2HPO_4 - KH_2PO_4 buffer, pH 7.0, and 0.01 M NaCl. Gas phase, N_2.

an initial burst or spike which is followed by a rapid decrease to a much lower steady-state O_2 evolution in the light. Exposing the chloroplasts with the same light again 3 minutes later results in a smaller O_2 spike than before. Aerobic chloroplasts also show the O_2-production spike but net O_2 uptake is usually seen in the steady state. An O_2 gulp is also seen upon darkening. The O_2-production spike is also lower in the second aerobic exposure (15). The decrease of the O_2-production spike in chloroplasts without added Hill oxidants is not due to a gradual deterioration of the chloroplasts since a dark interval serves to regenerate the O_2 spike again. de Kouchkovsky(16) has shown that the recovery of the O_2 spike is more rapid in air than under N_2.

An even more rapid recovery of the O_2-production spike is brought about by pre-illuminating the chloroplasts with far-red light. All the O_2 spikes shown in figure 7 were produced by the

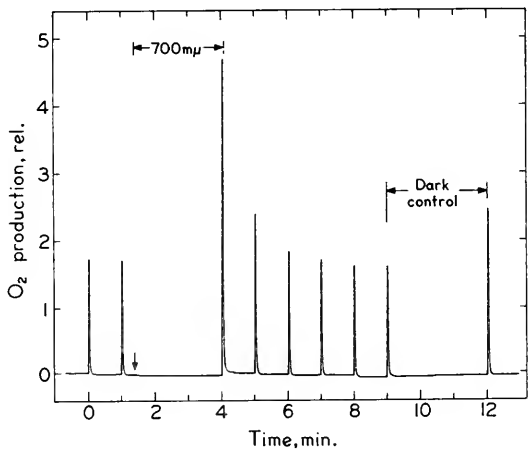

Fig. 7. O_2-production spikes produced by Swiss chard chloroplasts upon exposure to white light (5.2×10^4 ergs cm^{-2} sec^{-1}) filtered through 3 cm water and a Baltzers heat-reflecting filter (Calflex). The intensity of the 700 mμ light was 80 ergs cm^{-2} sec^{-1}. Chloroplasts equilibrated with the solution described in figure 7. Gas phase, N_2.

same 1.3-second white light exposure. In the first 3-minute interval between white flashes the chloroplasts were illuminated with weak (80 ergs cm^{-2} sec^{-1}) 700 mμ light for 2.6 minutes. No detectable O_2 exchange resulted from this 700 mμ exposure. How-

ever, when the 1.3-second white exposure was given again the O_2 spike had increased 2.77 times. A 3-minute dark interval, substituted for the 700 mμ exposure, increased the O_2 spike 1.49 times.

Butler[17] reported an opposing effect of 650 and 705 mμ light on the yield of chlorophyll fluorescence in a bean leaf. The constant fluorescence excited by a very weak 650 mμ light was greatly increased by a 5-second exposure to a much brighter 650 mμ light. After this treatment the weak light excited a continued high fluorescence which could be lowered to its former level by an exposure to 705 mμ light. If fluorescence can be equated to light energy which is wasted by the photochemical apparatus, then a parallel can be drawn between Butler's observations and the observations made on chloroplasts. Light at 650 mμ excites largely chlorophyll *b* and chlorophyll *a* 670. At low intensities this light can, because of overlap with the long-wavelength chlorophyll (the chlorophyll driving light reaction 1 of Müller, et al.[11]), sustain a low level of photosynthesis with little loss (low fluorescence). Chloroplasts without a Hill oxidant show a small amount of O_2 evolution in weak 650 mμ light. A 5-second exposure to bright 650 mμ light causes an accumulation of the product of the accessory pigment reaction (resulting in high fluorescence) since little energy can be absorbed by long-wavelength chlorophyll to drive the oxidation of the reduced accessory-pigment product (Müller, Weikard and Witt's[18,19] reduced plastoquinone-complex causing increased absorption at 515 mμ). Bright 650 mμ light gives a transient O_2-production spike which cannot be sustained for the same reason that the reduced product accumulates. A 700 mμ exposure which excites long-wavelength chlorophyll regenerates the oxidized plastoquinone-complex needed to produce another O_2 spike and allows weak 650 mμ light to be used more effectively (decreased fluorescence). A dark interval, shorter in air than N_2, regenerates material needed to produce another O_2 spike. Butler likewise found the increased fluorescence excited with bright 650 mμ light persisted longer in N_2 than in air.

Butler[20] has observed a shift to shorter wavelengths in the peak position of the action spectrum for the inhibition by far red of the fluorescence produced upon exposing a leaf to bright 650 mμ light. The peak of this action spectrum was shifted to shorter wavelengths when lower intensities of far-red light were used. A variation in the peak position from about 750 to 705 mμ occurred.

A similar effect of intensity was found in the action spectrum for the regeneration of the O_2 spike by far-red light. The action spectrum for the light-induced recovery of the O_2 spike was reported[15] to have a peak at 730 mμ. This action spectrum was determined with an intensity of 437 ergs cm^{-2} sec^{-1} at 700 mμ. When this action spectrum was determined with low intensities

(54 ergs cm^{-2} sec^{-1} at 700 mμ) the peak occurred near 700 mμ (figure 8). This shift of the peak position in the action spectrum for light regeneration of the O_2 spike may be due to competition between opposing reactions--a promoting reaction driven by far-red light and a depleting reaction resulting in O_2 evolution. With higher intensity far-red light some O_2 evolution would occur as a result of absorption by the tails of the absorption bands of the short-wavelength chlorophylls which extend to the near infra red.

The action spectrum for the O_2-production spike in figure 8,

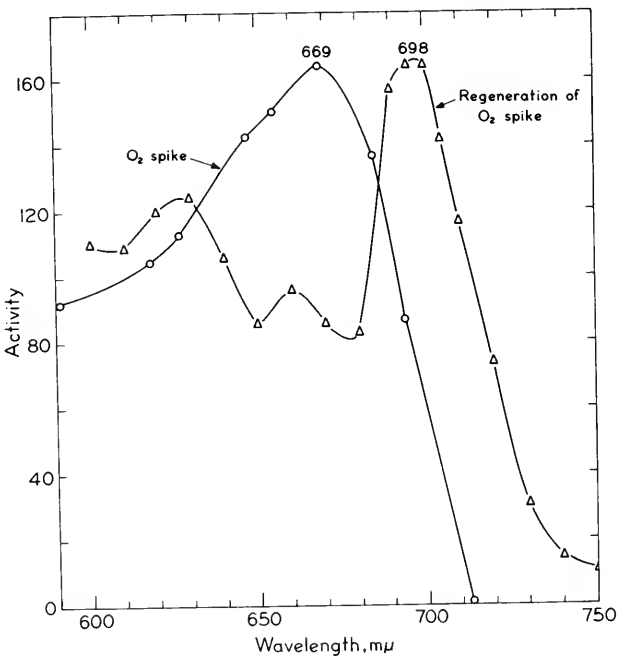

Fig. 8. Action spectra (equal incident quanta) for the O_2-production spike in background 700 mμ light and for the regeneration of the O_2 spike by light. Swiss chard chloroplasts equilibrated without Hill oxidants in the solution given in figure 6. Gas phase, N_2. The action spectrum for the regeneration of the spike is plotted above the level of the dark recovery. For this action spectrum the quantum flux at 700 mμ was 54 ergs cm^{-2} sec^{-1}. The same intensity 700 mμ background light was used for the action spectrum of the O_2 spike, while the energy of the 669 mμ beam was 475 ergs cm^{-2} sec^{-1}.

determined with weak 700 mμ background light, has a peak at 669 mμ and a far-red limit near 713 mμ. This action spectrum shows that the production of O_2 by chloroplasts illuminated with 700 mμ light is most effectively mediated by absorption by short-wavelength chlorophylls such as chlorophyll a 670 and chlorophyll b.

The action spectrum for the regeneration of the O_2 spike had a small hump at 660 mμ. The possibility that some accessory pigments also accompany the long-wavelength chlorophyll needs further investigation.

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LIGHT-INDUCED OXYGEN REACTIONS IN ISOLATED CHLOROPLASTS

Yaroslav de Kouchkovsky and Jean-Marie Briantais

INTRODUCTION

Studies of the kinetics of chlorophyll fluorescence [1] and of oxygen evolution [2] in intact Chlorella cells have suggested a cyclic mechanism involving three forms (A, B, C) of a "photocatalyst". Form A would accumulate in darkness and be converted into form B. By a first dark reaction, B would be converted to C with a concurrent evolution of oxygen. By a second dark reaction, A would be regenerated from C. If the rate constant of the second dark reaction is smaller than that of the first, the observed oxygen evolution transients in Chlorella cells can be explained. Up to recently [3], similar "induction" had not been found with chloroplasts [4]. Now, however, our work has shown that oxygen evolution in isolated chloroplasts is probably also based on a cyclic mechanism similar to that suggested previously. Moreover, our work has also shown that certain reactions associated with oxygen evolution by chloroplasts occur as well in the absence as in the presence of Hill reagents.

On the other hand, measurements of photosynthetic oxygen evolution are sometimes uncertain because of the possibility of an influence of light on respiration. For example, a measurement of quantum yield of photosynthesis may be in error if respiration, or other reactions of oxygen, have rates dependent on illumination.

These problems have led us to study the mechanism of photosynthetic reactions of isolated chloroplasts and to compare our findings with those for whole cells.

MATERIALS AND METHODS

Chloroplasts were isolated from Zea Mays. The methods of culture of the corn and of the isolation of chloroplasts are given elsewhere [5].

Hydrogen peroxide was detected colorimetrically using its reaction with titanium sulfate in strongly acid solution. Concentrations of H_2O_2 as small as 5 mμmoles/ml could be measured [5].

Photosynthetic activity was determined by following the oxygen concentration in an illuminated suspension. The oxygen concentration was measured

with a platinum electrode covered by a polyethylene membrane [5]. A concentration change of $\pm 10^{-8}$ M O_2 in < 2 sec could be measured.

Experiments were usually performed with saturating white light, at 15°C, and with suspensions equilibrated with air.

Abbreviations

DPIP : 2, 6 dichlorophenol indophenol; CMU : 3-(p-chlorophenyl)-1, 1-dimethylurea; FeCy : potassium ferricyanide; TMS : trismaleate buffer 0.1 M + sucrose 0.4 M.

RESULTS

Chloroplasts Without Hill Reagents

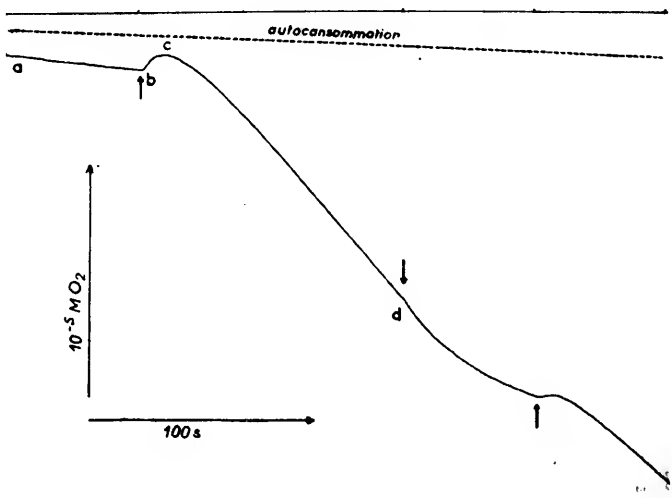

Fig. 1. Photographic reproduction of a recording of oxygen concentration changes with isolated chloroplasts in the absence of Hill reagents.
Chloroplasts: 300 µg chlorophyll/ml in TMS pH 6.6 . ↑ : light on; ↓ : light off. "Autoconsommation" : negative current, measured replacing chloroplasts by buffer. a, b, c, and d : see text.

Results of a typical experiment with chloroplasts in the absence of Hill reagents are shown in Fig. 1. In darkness, there is a very slow endogenous

uptake of oxygen (a-b), which we call "endoabsorption", because it differs from typical respiration [6]. When illumination begins, a "burst" of oxygen (b-c) appears, similar to bursts observed with Chlorella cells [2]. The burst is followed by a long-lasting, light-induced oxygen absorption (c-d) or "photoabsorption." On turning off the light, the rate of oxygen uptake abruptly increases (d); this increase in rate we refer to as "extra-absorption". Thereafter, the rate gradually declines, finally approaching asymptotically a constant value, corresponding to the rate of endoabsorption. When, after a dark period, illumination is repeated, a new, but smaller, burst appears. The amplitude of this second burst increases ("regeneration") with the length of the preceding dark period and may finally attain the amplitude of the first burst. These observations are similar to those of Fork [7].

These results, along with others to be described below, appear to be consistent with a modified cyclic mechanism:

$$4\ XHOHY \xrightarrow{k^*} 4\ XH + 4\ YOH \qquad (I)$$

$$4\ YOH \xrightarrow{k_1} O_2 + 2\,H_2O + 4\ Y \qquad (II)$$

$$4\ XH + 2\ O_2 \xrightarrow{k_2} 2\ H_2O_2 + 4\ X \qquad (III)$$

$$4\ X + 4\ Y + 4\,HOH \xrightarrow{k_3} 4\ XHOHY. \qquad (IV)$$

The mechanism supposes that there exists a limited amount of a complex (XHOHY) which disappears in light, giving an oxidant (YOH) and a reductant (XH). The oxidant would decay, giving O_2 and the "carrier" Y. The reductant would reduce molecular oxygen, giving hydrogen peroxide and generate the "carrier" X. Finally, X and Y would react with water regenerating the original complex. These different reactions have different rate constants (k^*, k_1, k_2 and k_3) and indicate that the sums of the different states of X and Y are constant and equal.

The present mechanism appears to be able to explain all the features observed in Fig. 1. First, the Mehler reaction [8] – equation III – along with the low activity of catalase in chloroplasts [5, 9] would explain the steady-state, net, consumption of oxygen in the light (hydrogen peroxide, the product of the Mehler reaction, was demonstrably formed in our experiments). Secondly, the transient net evolution of oxygen in the first seconds of illumination, and the later net consumption of oxygen, are readily explained if one supposes that reactions III and IV are slow compared with reactions I and II. Thirdly, the comparative slowness of reaction III means that a pool of XH must be maintained in light; it is the continuing oxidation of this pool which appears as extra-absorption (because at the end of illumination, reactions I and II, and hence oxygen evolution, stop). As the pool of XH is depleted during darkness, the oxygen consumption returns to that characteristic of endoabsorption. Fourthly, the gradual regeneration in darkness of a capacity to show a burst of oxygen in subsequent illumination would reflect the slow nature of reaction IV.

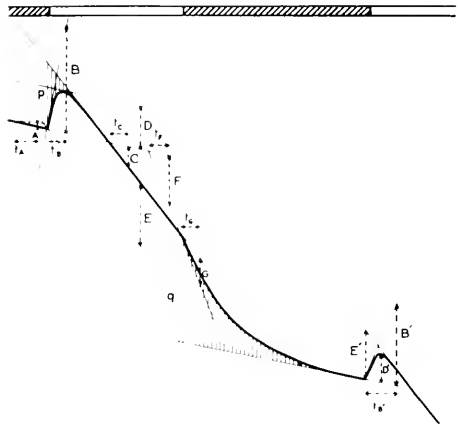

Fig. 2. Schematic representation of typical curves obtained with the "oxygen membrane electrode" (chloroplasts without Hill reagent).

——— : light; ▨ : dark. Abscissa : time; ordinate : oxygen concentration. See text for explanation of symbols.

A number of parameters defined in Fig. 2 serve to describe our experimental curves. The following parameters, or functions thereof, were useful in further analysis of our findings:

A/t_A = rate of the endoabsorption;
B/t_B = initial rate of the burst;
C/t_C = steady-state rate of the photoabsorption;
D = amount of oxygen evolved during the burst, assuming photoabsorption starts only after the end of the burst;
E = amount of oxygen evolved during the burst, assuming photoabsorption starts with the beginning of illumination (although this assumption may not be strictly correct, E is a better measure than is D; E was hence used for the quantitative estimations);
F/t_F = initial rate of the extra-absorption, calculated with the endoabsorption as reference;
G/t_G = initial rate of the extra-absorption, calculated with the photoabsorption as reference.

According to Mehler and Brown[10], both evolution and consumption of oxygen occur during photoabsorption. Then F/t_F will measure the rate of oxygen uptake and G/t_G the rate of oxygen evolution in the steady-state.

$B'/t_{B'}$, D' and E' are concerned with the second burst. The regeneration of the initial complex XHOHY (reaction IV) can then be evaluated as the ratio of, for instance, $B'/t_{B'}$ to B/t_B. When the value of this ratio was plotted as a function of the dark period between illuminations, a curve was obtained indicating that reaction IV is second order [5]. This is consistent with a reaction between two components (i.e., X and Y).

For the purpose of determining the kinetics of reaction II, the logarithm of the amount (p) of the oxygen precursor was plotted as a function of time. However, the first order rate constant previously reported [5] is certainly too small, because of the time response of our apparatus.

The rate of reaction III was analyzed by plotting the logarithm of the amount (q) of reducing power as a function of time. This reaction appeared to be first order, with a rate constant of $\sim 1.7 \cdot 10^{-2}$ sec^{-1}.
This reaction is probably bimolecular, but gives a first order kind due to the relatively high concentration of O_2 in aerobic conditions. Then, $k_1 \gg k_2$.

Several of our observations indicate that, in addition to the Mehler reaction there was another mechanism of light-dependent oxygen uptake. Firstly, CMU and ortho-phenanthroline, which are known to inhibit photosynthetic reactions, suppressed the oxygen burst, but did not entirely suppress all oxygen uptake in the light steady-state. Secondly, we found that net absorption of 1 mole O_2 was accompanied by the formation of only 1.5 mole H_2O_2, instead of the two moles anticipated in the Mehler reaction. Thirdly, when we plotted the rate of photoabsorption as a function of illumination, the resulting curve had a sharp break indicating two contributing reactions (in contrast, the illumination curve for the burst was a smooth curve rising to saturation). These findings are most simply explained assuming that a "photo-oxidation", as well as the Mehler reaction, contributes to oxygen uptake in the light [5].

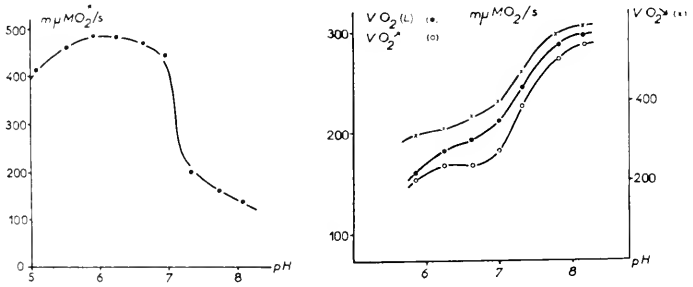

Fig. 3. pH effect on burst, photoabsorption and extra-absorption. Chloroplasts : 300 μg chlorophyll/ml in TMS. At left : initial rate of the burst (B/t_B, Fig. 2). At right : -●- = steady-state rate of the photoabsorption (C/t_C, Fig. 2); -o- = steady-state rate of oxygen evolution (G/t_G, Fig. 2); -x- = steady-state rate of oxygen uptake (F/t_F, Fig. 2).

The existence of the different postulated reactions was supported also by their different sensitivity to pH and temperature. The pH dependence of the initial rate of oxygen evolution during the burst, and the pH dependence of the steady-state rates of oxygen evolution and uptake during photoabsorption are shown in Fig. 3. The optimum for the burst is at pH ~ 6, while that for oxygen uptake is at pH ~ 8, similar to that for photophosphorylation [11, 12]. The difference in maxima between the burst and the steady-state oxygen evolution rests on the limitation of the total rate by the slowest reaction. Measurements as a function of temperature permitted a similar discrimination of the reactions [5].

Chloroplasts with Hill Reagents

It was of interest to try to detect an induction in the Hill reaction, although this has not been found previously [4]. Fig. 4 shows that, with ferricyanide, one can see a transient phenomenon very similar to the oxygen burst described above. But here, of course, the burst is followed by continuous oxygen evolution. On the other hand, with DPIP the steady-state is reached immediately, i.e., without an induction period.

Thus, different kinetics prevail in the Hill reaction with different oxidants. One can suppose that the difference in kinetics could depend on the occurence of only the photoreaction of "system 2" in the case of DPIP, and of both photoreactions ("systems 1 and 2") in the case of ferricyanide (cf. 7, 13).

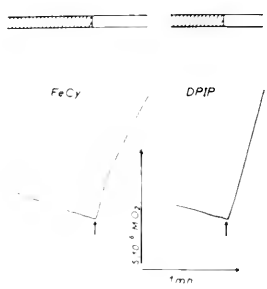

Fig. 4. Oxygen concentration as a function of time in Hill reactions with FeCy (10^{-4} M) and DPIP (5×10^{-5} M).
Chloroplasts: 315 μg chlorophyll/ml in TMS pH6.6.
↑ : light on.

If one continues to observe the Hill reaction, with ferricyanide [9] or with DPIP [14], one can note that, progressively, the rate of oxygen evolution decreases and finally attains a zero value. Afterwards, oxygen uptake occurs similarly to the photoabsorption already described in chloroplasts without Hill reagent. In Fig. 5, changes in oxygen concentration with and without

ferricyanide are shown. As the slopes of the curves during photoabsorptions indicate, light-induced uptake is lower when ferricyanide is present ($C_H < C$, Fig. 5). This may be explained by the existence of an equilibrium between the photoreduction of ferricyanide and the reoxidation of ferrocyanide. When the light is turned off, an extra-absorption appears which has a first order rate constant very close to that obtained without oxidant [9]. Analysis of the curves, in the manner already described, made it possible to distinguish a steady-state oxygen evolution (measured by G_H/t, cf. Fig. 2) and a steady-state oxygen absorption (measured by F_H/t, cf. Fig. 2). The origin of this oxygen absorption could be tentatively assigned to a Mehler reaction, which would compete with the Hill reaction. It is interesting to note that, as in chloroplasts without ferricyanide, hydrogen peroxide was detected and the ratio H_2O_2/O_2 was close to 1.5 [9]. This suggests again the occurence of a supplementary photo-oxidation.

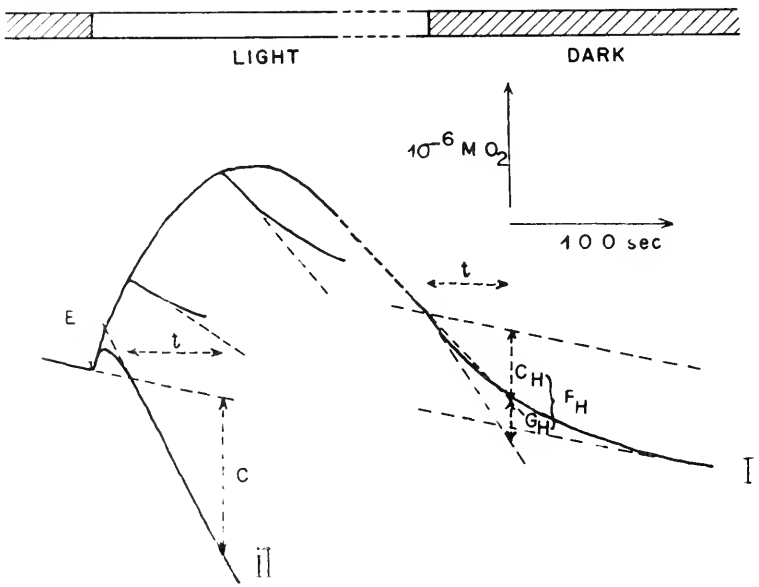

Fig. 5. Oxygen concentration changes in chloroplast suspensions with (I) and without (II) Hill reagent (FeCy : 10^{-4} M).
Chloroplasts : 315 μg chlorophyll/ml in TMS pH 6.6 ;
——— : light ; ////// : dark ; C, C_H, E, F_H, G_H : see text.

That a Mehler reaction can occur during the Hill reaction appears also from the following results. It was found that the decrease in the rate of oxygen evolution during the photoreduction of ferricyanide is accompanied by an increase in the rate of oxygen uptake (measured by the extra-absorption, as described above). This is partially shown in Fig. 5 by some segments of curves. Moreover, it was possible to distinguish two components in the value of the steady-state rate of oxygen evolution (G_H/t). If one assumes that, in the Mehler reaction, there is twice as much oxygen absorbed as oxygen evolved, then it is possible to subtract from the value of G_H/t (corrected for photo-oxidation) half of F_H/t, which represents the rate (V_M) of oxygen evolution by way of the Mehler reaction. The difference resulting from this subtraction will correspond to the rate (V_H) of oxygen evolution by way of the Hill reaction. On plotting the values of V_M as a function of V_H, a straight line was obtained [9].

$$4\ XHOHY \xrightarrow{k_L} 4\ XH + O_2 + 2\ H_2O + 4\ Y \qquad (V = I+II)$$

$$4\ XH + 4\ FeCy \xrightarrow{k_H} 4\ FeCy\ red. + 4\ X \qquad (VI)$$

$$4\ XH + 2\ O_2 \xrightarrow{k_M} 2\ H_2O_2 + 4\ X \qquad (VII = III)$$

Let us assume that the total amount (a) of the complex XHOHY + the reductant XH is constant. In our experiments, ferricyanide reduction will be second order ($V_H = k_H\ [XH]\ [FeCy]$), and the Mehler reaction will be first order ($V_M = k_M\ [XH]$). From this mechanism, one can derive the following reaction:

$$V_M = -\frac{k_M}{k_L + k_M}\ V_H + \frac{k_M\ k_L\ a}{k_L + k_M}\ ,$$

giving the expected linear dependence of V_M on V_H. Moreover, by graphical analysis, the maximum amount (a) of the complex XHOHY could be evaluated; the computed quantity was approximately the same as that determined from direct measurement of the burst (i.e., from the parameter E, Fig. 5). The numerical value of this quantity was 1 mole oxygen produced per 150 to 300 moles chlorophyll [5, 9].

DISCUSSION

It was possible to show that under certain conditions (in the absence of Hill reagents and in the presence of ferricyanide), induction occurs with isolated chloroplasts. As a result, the cyclic mechanism, previously postulated for whole cell photosynthesis, appears equally valid for at least some chloroplasts reactions in vitro. The exceptional case, i.e., the absence of induction with DPIP (at the concentration employed), can be understood assuming that with this reagent only "system 2" functions [13]. Photoreduction of the dye would then be faster than oxygen evolution.

The existence of an initial complex (the precursor of oxygen) was also demonstrated and its concentration estimated. This complex might contain plastoquinone, since hexane extraction not only cancels the steady-state oxygen evolution of the Hill reaction, but also the burst (although the photoabsorption – or at least that part which is a photo-oxidation – would remain in hexane-extracted chloroplasts)[14]. The oxygen burst, being a primary phenomenon of photosynthesis, the absence of this burst in hexane-extracted chloroplasts is in agreement with Witt's hypothesis that the photochemical act of "system 2" (linked to oxygen evolution) consists in the photoreduction of a plastoquinone[13].

It was also shown that measurements of oxygen evolved during the Hill reaction have to be corrected for the interfering Mehler and photo-oxidation reactions. Light-induced oxygen absorption was also found with intact Chlorella cells, but its mechanism seems to be more complicated[9].

Employing the proposed mechanisms it has been possible to generate data mathematically similar to those found in our experiments. Some curves, computed in this way, are shown in reference 5.

Acknowledgement

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STUDIES WITH FLASH ILLUMINATION ON THE ENHANCEMENT EFFECT IN CHLOROPLASTS

C.P. Whittingham and P.M. Bishop

Emerson and Lewis (1) were the first to observe in Chlorella that the quantum efficiency of photosynthesis in far-red light was very low. This was particularly surprising because whilst the absorption curve was more or less symmetrical about the 680 mμ peak, the action spectrum was asymmetrical. Many years later Emerson et al. (2) showed that the efficiency in the far-red could be increased if the cells were simultaneously illuminated with a second shorter wavelength. The action spectrum for the increased rate of photosynthesis resulting from a second wavelength superimposed on a beam of light of 697 mμ showed two peaks, one at 650 and one at 670 mμ. The peak at 650 mμ is characteristic for absorption by chlorophyll b, and Emerson concluded that the simultaneous excitation of chlorophyll b must improve the photosynthetic efficiency of the light absorbed in the far-red by chlorophyll a. It was observed later that the enhancement effect resulted from excitation of a second pigment throughout its spectrum. For example, excitation in Chlorella of either the blue or the red absorption bands of chlorophyll b was equally effective for enhancement. The general conclusion was that it was necessary to have simultaneous excitation of both chlorophyll a and of some other pigment for efficient photosynthesis. In studies with monochromatic light, the relative inefficiency of absorption by chlorophyll a alone appears only at the far-red end of the spectrum because this is the only region in the visible where chlorophyll a is the sole absorbing pigment.

Emerson made no comment on the second peak at 670 mμ in the enhancement action spectrum observed by him. A peak (Govindjee, et al. (3)) or shoulder (Myers and French (4)) at 670 mμ in the action spectra for the Emerson effect in Chlorella was interpreted by the first mentioned authors as an indication of the participation in the enhancement effect of a chlorophyll a type with a maximum absorption at 670 mμ. Emerson and Rabinowitch (5) (c.f. also Franck (6)) proposed the following hypothesis. Two photochemical reactions occur in photosynthesis; one of these is caused by non- or weakly- fluorescent chlorophyll a, the second

by a fluorescent chlorophyll a. Excitation of the non-fluorescent chlorophyll a alone does not lead to photosynthesis. However, if both chlorophylls are excited simultaneously, the two reactions can cooperate, and lead to an enhanced photosynthesis. An increased photosynthetic activity (enhancement effect) due to simultaneous illumination by two different wavelengths has now been shown in a large number of organisms. The so-called accessory pigments, e.g. chlorophyll b in Chlorella, and the phycobilins in red and blue-green algae, effect photosynthesis by transferring their excitation energy by resonance transfer to fluorescent chlorophyll a but not to the non-fluorescent form.

Myers and French (7) have shown that the enhancement effect observed with Chlorella illuminated with two wavelengths does not require that the two wavelengths be given simultaneously, but that these may be given alternately in periods of several seconds duration. This suggested the formation of an intermediate common to the two reactions with a life of several seconds. Such a mechanism could also be used to explain the observations of Whittingham and Brown (8), which extended those made earlier by Allen and Franck (9). They observed, with Ankistrodesmus, that the photosynthetic yield of oxygen from a light flash of 35 msec. duration was increased if it was preceded by a flash of 100 μsec. The yield from the pair of flashes varied with the interval separating them, rising at 20° C to a maximum when the interval was 0.7 sec. and decreasing as the time separation of the flashes was increased, until after 10 or 15 sec. little enhancement was observed. Further work by Whittingham and Bishop (10) has shown that when the temperature was reduced to 4° C, the maximal yield for a long flash preceded by a short flash was of the same order of magnitude as the maximal yield of the same two flashes at 20° C, except that the time between the flashes for maximum yield was now 16 sec.

At 4° C it was possible to investigate these flash effects with chloroplasts isolated from spinach leaves, using different oxidant systems. These consisted of:-

a) basic ferricyanide - containing, in a total volume of 2.3 ml. potassium ferricyanide 2 mμ moles; Tris-HCl buffer at pH 7.4 40 μmoles; sodium chloride 70 μmoles; magnesium chloride 10 μmoles.

b) ferricyanide with phosphorylating reagents - containing, in addition to the basic medium, 2 μmoles ADP and 40 μmoles inorganic phosphate.

c) ferricyanide with an uncoupling reagent - in which ammonium chloride was added to the basic medium to give a final concentration of 0.01M.

d) ferricyanide with catalytic amounts of TPIP - in which 0.01 μmoles of TPIP (2:3-6-trichlorophenol-indophenol) were added to the basic medium.
e) TPIP - in which the potassium ferricyanide in the basic medium was replaced by 0.1 μmoles of TPIP.

Oxygen output from the chloroplasts was determined by a flow system, using a modified version (11, c.f. also for details of materials and methods) of that described by Whittingham (12). The method consists essentially of the use of a carrier gas (oxygen free nitrogen) to sweep continually through the chloroplast suspension and then over an oxygen measuring galvanic cell. The output from this cell was amplified and recorded, and the yield of oxygen from the flash illuminated chloroplasts determined from the area under the recorded curve.

In experiments using white light, short flashes of 100 μsec. were generated by condenser discharge through a xenon discharge tube. Long flashes of 35 msec. duration and of sufficient intensity to achieve flash saturation, were produced from Photographers' flash bulbs. For monochromatic flashes, a flash bulb was used in conjunction with an interference filter with transmission either at 697±7 mμ or 644± 13 mμ. Continuous background light was passed through a small grating monochromator with slit widths adjusted to give a beam of 5 mμ band width. Over the wavelength range used there was a slight variation in light intensity from 7.4×10^{-4} watts/cm^2 at 645 mμ to 6.0×10^{-4} watts/cm^2 at 700 mμ.

In the arrangement of an experiment using white light flashes, a single long flash was always fired first, and then as every third illumination type thereafter. The yield plotted against time showed the rate of decay of chloroplast activity.

When assessing the effect of continuous supplementary light, the chloroplasts were illuminated by a monochromatic beam until steady state photosynthesis was reached, as shown by the plateau on the record chart. A flash was then superimposed on this background; the supplementary light was not turned off until the steady state background level of oxygen production was again reached. After a dark interval, during which the oxygen was flushed out of the system, another monochromatic background wavelength was used for illumination and the process was repeated.

In the third type of experiment, the monochromatic background light was separated in time from a monochromatic flash. Here, only two different wavelengths were used, a 653 mμ background

together with a 697 mμ flash, and a 700 mμ background in conjunction with a 644 mμ flash. The yield from a single flash, after a long dark period, fired at the beginning of the experiment and at regular intervals during the course of it, was again used as a measure of the decline in chloroplast activity.

Effect of a single short flash

No oxygen yield was obtained from a single short flash from chloroplasts reducing TPIP. Witt et al. (13) using a rapid spectrophotometric assay, showed that TPIP was reduced by chloroplasts instantly within a flash of 10 μsec.; reduction was followed by rapid partial re-oxidation of the dye in the dark. In order to estimate the amount of TPIP reduced in a single flash, several flashes had to be given, and Witt calculated that, in a single flash, two molecules of dye were reduced; this corresponded to the evolution of one molecule of oxygen per 12,000 chlorophyll molecules. It seems possible, therefore, that the amount of oxygen liberated by a single flash of 100 μsec., originally very small, is reduced by competing back reactions to an amount which is below the limits of detection of the measuring apparatus.

A very small oxygen yield was obtained from a single short flash when catalytic amounts of TPIP were present together with ferricyanide. Presumably the TPIP reduced photochemically by the chloroplasts in the light, is reoxidixed chemically more rapidly by the substrate amounts of ferricyanide present, than by molecular oxygen, so that a measurable amount of oxygen is released.

No oxygen yield was obtained from a single short flash with chloroplasts and ferricyanide, either on its own, or with the addition of phosphorylating or uncoupling agents. The absence of a fast re-oxidation of the ferricyanide reduced by chloroplasts in the light, has been reported on several occasions. It seems possible, that in the case of ferricyanide no oxygen is produced as a result of a single short flash.

The production of oxygen from a single short flash thus results when chloroplasts are reducing TPIP, or ferricyanide with catalytic amounts of TPIP; this is in contrast to the situation occurring with ferricyanide alone since then no oxygen yield from a single short flash is apparent.

Pre-excitation by a short flash on the yield of a following long flash

Oxygen was produced as a result of a single long flash and a brief flash was found to increase the yield from a long flash in

all the systems studied. In the case of chloroplasts reducing ferricyanide and TPIP, where oxygen was produced from the single short flash itself, the yield from the pair of flashes was considerably greater than the sum of the yields from the two flashes when these were given separated by a very long dark interval.

The optimum dark period between the two flashes at which maximum yield was obtained from the second flash, varied according to the oxidant used, as can be seen from figure 1.

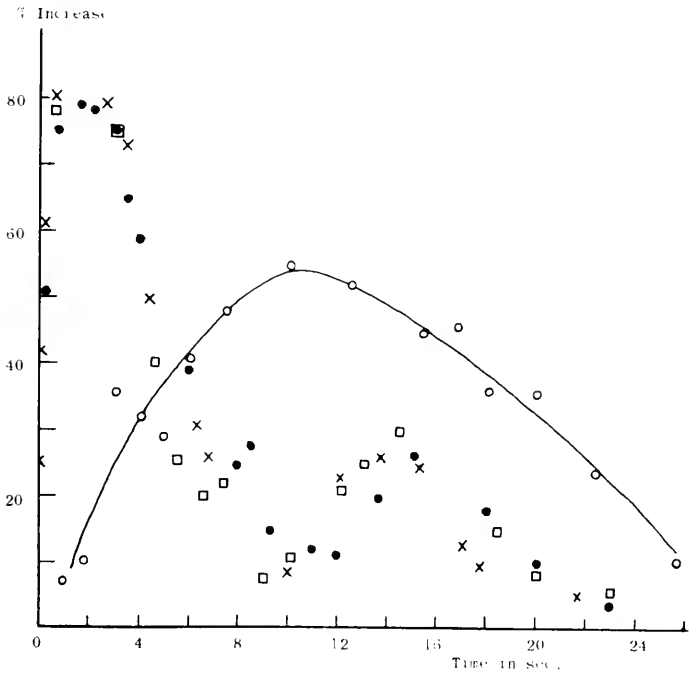

Figure 1 Relationship between the combined yield of oxygen for a short and long flash as a function of the dark time separating the two flashes. The yield is expressed as percentage increase over the yield of the long flash given alone.

- ○ ferricyanide with phosphorylating reagents
- ● ferricyanide with ammonium chloride
- □ ferricyanide with catalytic amounts of TPIP
- × TPIP

The data obtained with chloroplasts reducing ferricyanide

shows that, as the dark period between the two flashes is increased, the yield from the long flash rises slowly to a maximum; this occurs when the two flashes are about 10 sec. apart. The yield remains relatively constant up to 15 sec. separation, and then declines gradually. There is still some influence of the first flash on the second when the two are 25 sec. apart. A very similar plot is obtained when ferricyanide is reduced in the absence of phosphorylating reagents.

As may be observed from fig. 1, the addition of an uncoupling agent to a ferricyanide system has the effect of shortening the dark time necessary between the two flashes to obtain the maximum yield from the second flash. The yield from the long flash now rises rapidly as the dark time between the short and the long flash is increased; maximum yield is obtained when the two are between 1.5 and 2.5 sec. apart. With further increase of the dark time between the flashes, the yield declines exponentially to about 10 sec. separation time; following this there is a further slight rise with a maximum at 15 sec. and then a further decline, until at 20 sec. there is only a slight effect of the first flash on the second. Figure 1 also shows that very similar results are obtained when catalytic amounts of TPIP are added to ferricyanide and when TPIP alone is used as an oxidant. The maximum increase in the yield of the long flash is obtained when the two flashes are between 0.5 and 3 sec. apart; there is a similar secondary peak occurring when the two flashes are separated by a dark interval of about 14 sec.

The data presented above demonstrate that the time course of the flash effect varies according to the nature of the oxidant system. The results can be separated into two groups according to whether the maximum yield is obtained when the two flashes are separated by a long time interval - as found with ferricyanide in the presence or absence of phosphorylating reagent, or by a short one - as occurs with TPIP and on the addition of TPIP or of an uncoupling agent to the ferricyanide system. The size of the increase at its maximum is largest in the latter group.

Monochromatic flash illumination with monochromatic continuous background light

The results obtained from a typical series of experiments with chloroplasts reducing ferricyanide in the presence of phosphorylation cofactors, are presented in figure 2. From the figure it can be seen that there are two regions where background light is most effective in enhancing the yield from the 697 mμ flash; one extending from 642 to 660 mμ, has a broad peak from 646 to 653 mμ;

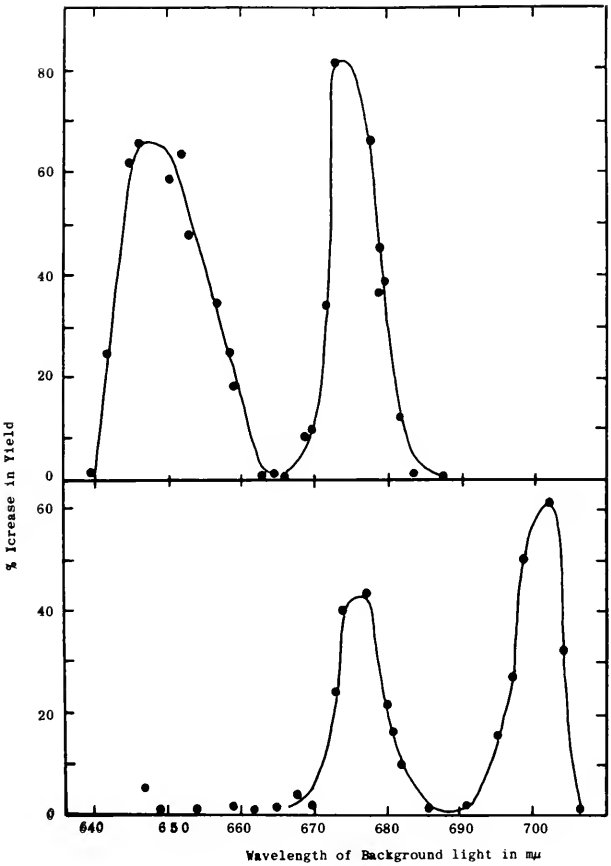

Figure 2 Percentage increase in yield for a monochromatic flash as a function of the wavelength of the continuous background light. The percentage is expressed as the increase in yield compared to a flash given in darkness. The upper figure was obtained with a 697 mμ flash, the lower with a 644 mμ flash.

a second region from 671 to 683 mμ has a sharp peak at 675 mμ. Beyond 685 mμ, and in the trough between the two peaks, the addition of background light appears to have little influence on the yield of the flash.

The results from the converse experiment, using a 644 mμ flash are shown in figure 2. Here also there are two regions where the background light is effective in enhancing the yield from the flash. These extend from 670 to 678 mμ, with a sharp

peak at 675 mμ; and the second from 690 and beyond to about 705 mμ, with a peak at 700 mμ. There is little or no influence of the background light on the flash from 642 to 670 mμ, and between 680 and 690 mμ.

The magnitude of the enhancement was found to be slightly greater with a 697 mμ flash than with a 644 mμ flash. A reduction in the intensity of the background light reduced the peak heights at the enhancing wavelengths, but did not alter their position: this effect was considerably greater with the 697 mμ flash than with the 644 mμ flash. It seems probable that the use of a higher intensity background light would have increased the absolute peak heights of enhancement obtained with a 697 mμ flash.

When an uncoupling agent was added to ferricyanide it was still possible to obtain an enhanced yield from both flashes, by the use of background light. The yield from the 697 mμ flash was increased 70% with a background of 653 mμ, and that of a 644 mμ flash 40% with the addition of a 700 mμ background.

With TPIP alone, at the concentration used in the white light flash work reported above, there appeared to be no effect of background light on the yield of either of the flashes. However, a reduction in the concentration of the dye from 3×10^{-4}M to 5×10^{-5}M, resulted in an increase in yield of 55% from a 697 mμ flash with a 653 mμ background; with a 675 mμ background the effect could not be established as significant. No increase was obtained with a 644 mμ flash, and when this was superimposed on a 700 mμ background, of the same intensity as normally used, a reduction in the yield occurred, compared to the flash without background light. This inhibitory effect could be eliminated by a reduction in the intensity of the 700 mμ illumination.

The data thus show that it is possible to enhance the oxygen yield from a flash of light by the addition of supplementary light of certain wavelengths. The extent of the enhancement obtainable depends on the oxidant the chloroplasts are reducing. With ferricyanide, the output from a 697 and a 644 mμ flash can both be increased; the marked dependence of the enhancement on the wavelength of the background light indicates the presence of different forms of chlorophyll with sharp absorption bands. When an uncoupling agent is added to ferricyanide, it is still possible to enhance the yield from both flashes in conjunction with background wavelengths found to be effective with ferricyanide alone. With TPIP as oxidant, however, it is only possible to enhance the yield from the long wavelength flash; there is some indication

that a concentration effect may also be concerned, since it is not always possible to obtain enhancement.

Flash illumination following continuous background illumination

The data obtained from a series of experiments with chloroplasts reducing ferricyanide with phosphorylating cofactors present, in which the flash was separated from the background light by a finite dark interval, is shown in figure 3. The lowercurve on the graph shows the results obtained with a background light of 653 mμ and a flash of 697 mμ. The yield from the flash increases as the time between the termination of the background

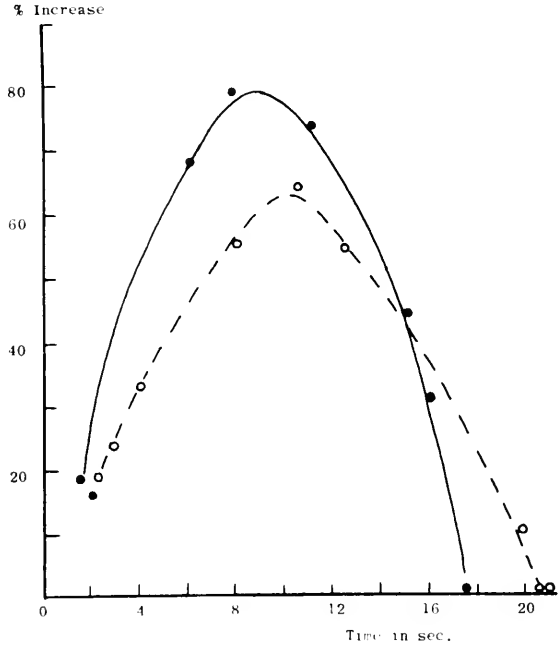

Figure 3 Percentage increase in oxygen yield per flash as a function of the time interval between the cessation of the background light and the flash. The percentage is expressed as the increase in yield compared to the flash given in darkness.

O Yield from 697 mμ flash following illumination at 653 mμ

● Yield from 644 mμ flash following illumination at 700 mμ

light and the flash is increased until maximum yield is obtained when the two are separated by 8 - 10 sec. With further increase of the dark time, the yield falls off rapidly until there is no further influence of the background light on a flash given after a 20 sec. dark period.

The results of the converse experiments, with a background light of 700 mμ and a flash of 644 mμ are shown in the same figure. Maximum yield from the flash is obtained when a dark interval of 7 - 11 sec. intervenes between the background light and the flash. The yield decreases rapidly as the dark period is increased beyond 12 sec. The size of the increase in yield at its maximum is greatest with this combination of flash and background light.

The data thus show that a background light which enhances the yield of oxygen from a flash when given simultaneously, can be separated in time from the flash and still produce enhancement. The time course of this effect is very similar whether the background is at 700 mμ and the flash is at 644 mμ, or the background is at 653 mμ and the flash at 697 mμ.

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SOME EFFECTS OF MONOCHROMATIC LIGHT ON OXYGEN EVOLUTION AND CARBON DIOXIDE FIXATION IN CHLORELLA PYRENOIDOSA

Max H. Hommersand

One of the problems that is encountered in investigations of chromatic transients and enhancement is that closely related species or varieties of algae often differ in their responses to treatment with monochromatic light, even when they appear to possess essentially the same pigment complex. Recently, we have investigated material of Chlorella pyrenoidosa, Emerson strain, obtained from the Indiana University Algae Culture Collection, #252. Our cells show chromatic transients having the same general magnitude and wavelength characteristics as those described in Chlorella pyrenoidosa by Myers and French[1], but, so far, we have failed to observe any sustained enhancement. Enhancement was looked for both in polarographic studies on oxygen evolution, and in experiments on the rate of fixation of labeled carbon dioxide in monochromatic light. In addition to chromatic transients, our material of Chlorella produces a large, negative oxygen transient immediately after the light is turned off. This transient has the same general shape as the one described by French and Fork[2] in Porphyridium, and referred to by them as photostimulation of respiration.

Light-dark oxygen transients.

We have measured light-dark oxygen transients in Chlorella polarographically under conditions in which the base-line drift was less than 3% of full scale per hour, and in which the steady-state photosynthesis rate was maintained at the same level at all wavelengths. A regimen was adopted in which the cells were given monochromatic light for six minutes followed by fourteen minutes of darkness. This allowed time for the photosynthetic rate to become stable at all of the wavelengths tested, except possibly in the region around 480mμ. The dark period was sufficiently long in most cases to allow the transient to return to the base line. The negative transient varied linearly as a function of the steady-state photosynthesis rate throughout the visible spectrum. It was observed that the size of the transient was the same, within experimental error, at all wavelengths when light

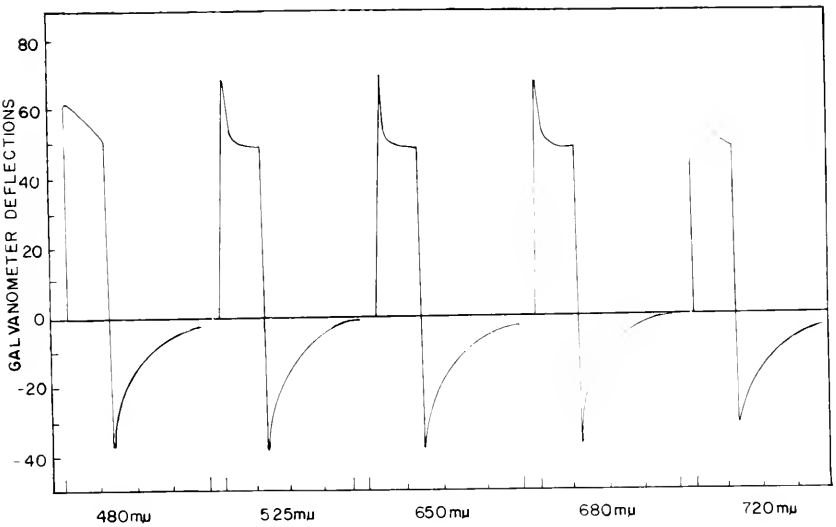


Fig. 1. Oxygen transients measured at five different wavelengths. Monochromatic light of 6.6 mμ half-band-width was provided from a Bausch & Lomb monochromator. The curves are plotted from galvanometer readings taken every fifteen seconds. Other details in the text.

intensities were used which gave the same steady-state photosynthesis rate at each wavelength. This was even true of transients measured in the far-red region. Some selected examples are shown in figure 1. The transient at 720mμ is particularly interesting because of its similarity to the transients occurring in the visible region. Since oxygen evolution in untreated whole cells probably requires the participation of two pigment systems, it appears that in this strain there is relatively good overlap in absorption by the pigments of the two systems even at relatively long wavelengths.

The details in the shape of a light-dark oxygen transient are repeatable when a series of such transients are measured at a particular wavelength. If, however, at the end of the period of a transient the wavelength of the light is shifted and the light intensity at the new wavelength is preset to give the same steady-state photosynthesis rate, then a new transient having a

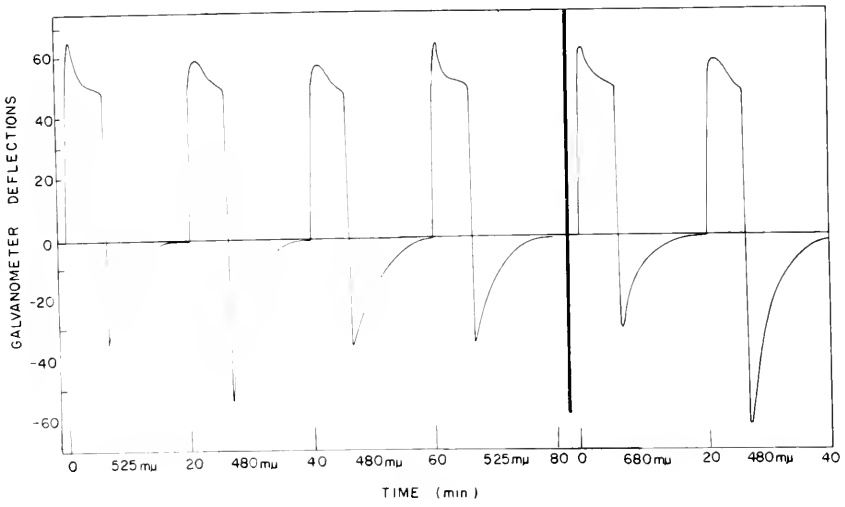
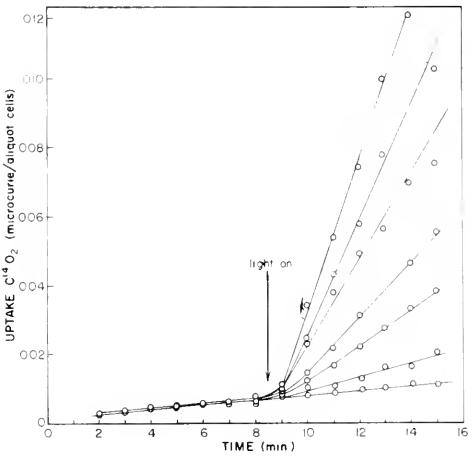


Fig. 2. Sequence of oxygen transients 525mμ → 480mμ → 480mμ → 525mμ (left side) and 680mμ → 480mμ (right side).

different magnitude from the previous one may be generated. The greatest changes in the magnitude of negative transients have been observed in shifts from a wavelength where the primary pigment system (System I of Duysens[3]) is the principal light absorber to a wavelength where the secondary pigment system (System II of Duysens) is the principal light absorber. Two such shifts (525mμ → 480mμ; 680mμ → 480mμ) are shown in figure 2. After a single aberrant transient has been produced, all succeeding transients are normal for the particular wavelength. In the reverse shift, from a wavelength where the pigments of System II predominate to one where the pigments of System I predominate, in this case from 480mμ → 525mμ, or from 480mμ → 680mμ, no temporary change in the behavior of the transient is observed. I would interpret these results as indicating that an adjustment takes place, either in the relative efficiency in the utilization of energy trapped by the two pigment systems for photosynthesis, or in the balance between photosynthetic and respiration rates, when a shift is made from one wavelength to another. This adjustment is probably of such a nature as to tend to maximize the quantum yield. The adjustment probably takes place rapidly



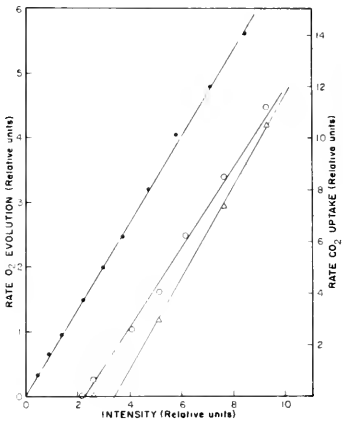


Fig. 3 (left). Uptake of $C^{14}O_2$ at different intensities of far-red light compared to the rate of uptake in the dark. A Wratten sharp-cut filter #45 (1% transmission at 705mμ) backed with a Corning filter #2418 was employed. Relative intensities were measured with a Kipp thermopile and the data are all plotted on the same intensity scale.

Fig. 4 (right). Comparison between the net rate (light rate minus dark rate) of CO_2 uptake and O_2 evolution in far-red light as a function of the light intensity, showing an intensity threshold for CO_2 fixation. CO_2 uptake was measured as described in figure 3 and O_2 evolution was followed polarographically. The relation between the slopes of the curves is arbitrary. The curves are: ●, oxygen evolution; o, Δ, CO_2 fixation (two experiments).

when the shift is to a region where the pigments of System I predominate, and is slow--slow enough to be reflected in the transient--when a shift in the opposite direction occurs. The conditions imposed at one wavelength which affect the magnitude and shape of a transient at another wavelength appear to be dissipated slowly over a period of several hours in the dark.

Max H. Hommersand

Threshold light intensities for carbon dioxide fixation.

In general, the photosynthetic rate measured on an oxygen electrode is linear as a function of light intensity over a wide range of intensities. Non-linearities have been observed with the oxygen electrode in Chlorella in the region around 700mμ by Myers and Graham[4]. In our strain of Chlorella, oxygen evolution appears to be linear as a function of intensity in the far-red region and does not show any threshold at low light intensities (fig. 4).

An examination of the rate of carbon dioxide fixation at wavelengths longer than 710mμ using labeled bicarbonate showed that while CO_2 uptake is linear as a function of light intensity over most of the intensity range, it has a distinct threshold at moderately low light intensities (figs. 3 & 4). If one uses either younger cells, or successively longer wavelengths of far-red light, this intensity threshold rises rapidly to a point where very bright light is needed to effect a light promoted fixation. We have chromatographed the products of $C^{14}O_2$ fixation at different light intensities given for different lengths of time, and have found that at intensities at or below the threshold the distribution pattern of the label is the same as in darkness. At intensities only 10-15% greater than the threshold intensity a labeling pattern characteristic of CO_2 fixation in the light, in which sugar mono- and di-phosphates, alanine, serine, glycine, and sucrose are rapidly labeled, is discernible.

Effects of carbonyl cyanide m-chlorophenylhydrazone (CCCP) on oxygen evolution.

As can be seen in figure 5, CCCP does not have any significant inhibitory effect on the steady-state, in-vivo photosynthesis rate in Chlorella cells over a wide range of concentrations (5×10^{-6}M to 10^{-4}M) in which CCCP stimulates respiration several fold, presumably by uncoupling oxidative phosphorylation. CCCP does, however, induce a lag in the onset of oxygen evolution, the length of which varies depending on light intensity, light quality, and on the concentration of CCCP (figs. 5 & 6). The lag observed in the induction of oxygen evolution is significantly more sensitive to CCCP for photosynthetic rates above compensation, than it is for rates below compensation. At high CCCP concentrations (5×10^{-5}M) and low light intensities (less than 1000 ft. candles) even the onset of photosynthesis below the compensation level may be affected. By using various combinations of colored filters, and by adjusting the relative light intensity

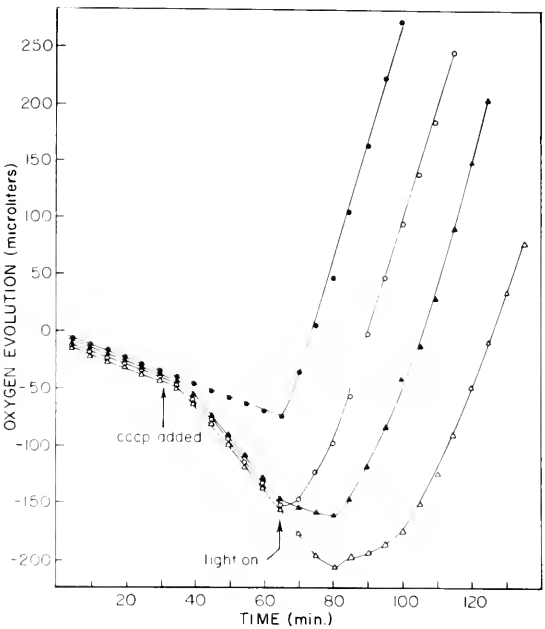


Fig. 5. Effect of CCCP on respiration and photosynthesis in Chlorella pyrenoidosa. Pardee buffer[5] adjusted to provide a constant 1% CO_2 tension was used in the center well of the Warburg vessels. The light intensity was 1500 ft. candles. The curves are: ●, control; o, 10^{-5}M CCCP; ▲, $3x10^{-5}$M CCCP; △, $8x10^{-5}$M CCCP.

to obtain approximately the same photosynthetic rates in the controls, the relative effectiveness of different colors of light in overcoming the photosynthetic induction lag caused by CCCP was compared. It was observed that under the experimental conditions, red light at wavelengths longer than 660mμ was the most effective in overcoming the lag caused by CCCP. White light, adjusted to an intensity that gave the same photosynthetic rate in the controls, was much less effective than red light, and blue light was still less effective than white light (fig. 6).

It is surprising, in view of the fact that CCCP is known to be such an effective uncoupler of photosynthetic phosphorylation in isolated chloroplasts of higher plants[6], that this compound

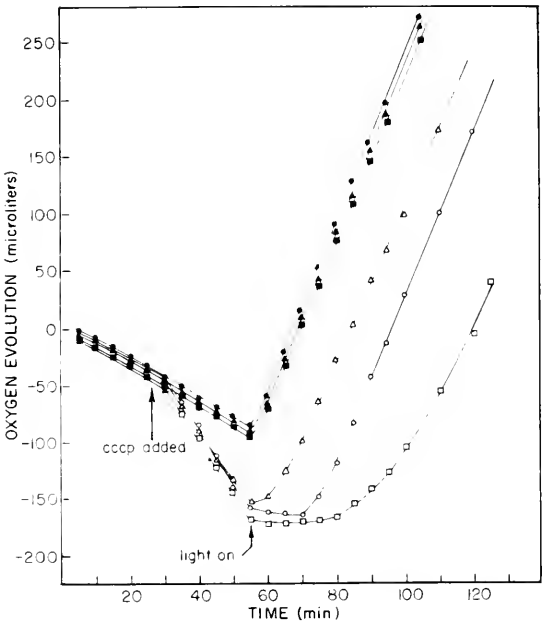


Fig. 6. Effects of different colors of light in overcoming the induction lag caused by treatment with CCCP. The curves are: ●, white light, control; o, same, 3.3 x 10^{-5}M CCCP; ▲, red light (Corning filter #2030), control; △, same, 3.3 x 10^{-5}M CCCP; ■, blue and far-red light (Corning filter #5031), control; □, same, 3.3 x 10^{-5}M CCCP.

does not inhibit photosynthesis in living cells. There is an indication, however, that CCCP may uncouple some of the photosynthetic phosphorylation that occurs in-vivo in algae. We have followed the effect of CCCP on the uptake of labeled acetate in Chlamydomonas reinhardtii, a species in which light promotes acetate assimilation several fold above the dark rate. (CCCP causes an induction lag in oxygen evolution in Chlamydomonas the same as in Chlorella.) Acetate uptake was completely inhibited by CCCP in the dark at concentrations below 10^{-5}M, and the inhibition was not in any degree reversed by light (fig. 7). By comparison, cyanide is much less toxic.

It is generally held by most investigators that the light

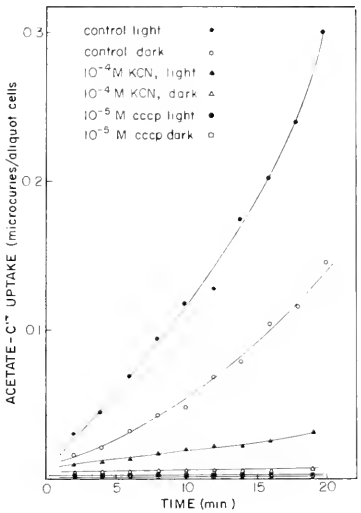


Fig. 7. Uptake of sodium acetate-1-C^{14} (3×10^{-4}M) by Chlamydomonas reinhardtii in light and dark, and in the presence of KCN or CCCP as indicated. Far red illumination was provided from two sides by 'sun-gun' lamps at a total intensity of 15,000 ft. candles each passed through a Wratten #45 filter backed by a Corning red sharp-cut filter. The cells were pretreated for 20 minutes under the experimental conditions before the label was added.

promoted uptake of acetate is dependent on the production of ATP by photophosphorylation. If this is so, then photophosphorylation resulting in the formation of ATP that is consumed outside the chloroplast must be CCCP sensitive. Conversely, the additional ATP involved in light promoted acetate uptake may possibly come from light-stimulated respiration, and the effect of CCCP would then be due to an uncoupling of this respiration. We are conducting experiments to test these alternative possibilities. In any event, a CCCP-insensitive photosynthetic pathway probably exists in living cells, and is lost during their disruption in the preparation of isolated chloroplasts.

Since the uncoupling of oxidative phosphorylation by CCCP would have the effect of increasing the steady-state ADP/ATP ratio in the cell, it may be that the induction of photosynthesis requires moderately high levels of ATP that are lacking in cells treated with CCCP. It has been shown that in isolated chloroplasts far-red light is more effective in the synthesis of ATP than visible light[7]. Our experiments on the effectiveness of color and intensity of light in overcoming the induction lag in oxygen evolution caused by CCCP can also be interpreted as evidence that more ATP is synthesized in far red than in visible light. Our data are in agreement with the suggestion that there is a higher rate of ADP consumption and ATP production at wavelengths where absorption is predominantly by pigments of System I, and, correspondingly, that there is a relative higher rate of ADP

production and ATP consumption at wavelengths where pigments belonging to System II absorb predominantly.

Conclusions.

The relationships between enhancement, chromatic transients, and transients attributable to photorespiration are difficult to assess. Enhancement appears to depend in some way on the fact that absorption by the pigments of System I is not always effectively overlapped in the far-red region of the spectrum by absorption due to pigments of System II. Where enhancement is absent, as in our strain of Chlorella, the overlap of the two pigment systems appears to be better than usual, and the photosynthetic rate does not decline rapidly in the far red.

Chromatic transients appear to be a measure of the difference in the balance achieved in the utilization of light energy absorbed by the two pigment systems, depending on the color of light the cells are receiving. The largest transients produced by wavelength shifts reflect the greatest differences in the relative participation of the pigment systems in providing energy for steady-state photosynthesis. The respiration rate may play a role in achieving this balance, and all factors probably combine to sustain the maximum quantum yield.

Light-dark transients have been interpreted both as a photostimulation of respiration and as oxygen uptake in the reoxidation of intermediates of photosynthesis inside the chloroplast. In either case, changes in the magnitude of these transients resulting from wavelength shifts provide a measure of the rate at which already-formed patterns of energy transfer or utilization are broken down and new ones are established.

This investigation was supported by a research grant GB-424 from the National Science Foundation.

Credit is due to Mr. Douglas Fambrough for his work on the effects of far-red light on carbon dioxide fixation reported in this paper. The excellent assistance of Miss Mary Thomas and Mr. Carl Lundeen is also gratefully acknowledged.

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ILLUMINATION DEPENDENCE OF ENHANCEMENT

T. T. Bannister and M. J. Vrooman

The subject of this paper is the dependence of enhancement in Chlorella pyrenoidosa str. 3, on short-wave (482 mμ) and far-red (696 mμ) illuminations. Two enhancement functions were determined, the enhancement (E) and the excess rate (D):

$$E = (p_{12}-p_1)/p_2 \qquad (1)$$

$$D = p_{12}-p_1-p_2 \qquad (2)$$

Here p_1, p_2, and p_{12} are the steady state photosynthesis rates in short-wave, far-red, and in the two lights, respectively. To facilitate reference to illumination curves of photosynthesis, p_1, p_2, p_{12} and D were expressed as rates relative to the saturated rate in white light. The observed functions $E(I_1, I_2)$ and $D(I_1, I_2)$ were compared with these predicted by the "spill-over" and "separately packaged pigment" models of enhancement. The predicted functions, which are the same for both models, explain many, but not all, characteristics of the observed functions.

METHODS

Oxygen evolution rates were measured with a horizontal platinum electrode similar to that of Myers and Graham [1]. The platinum surface on which the cells were sedimented lay about 0.5mm below a dialysis membrane which separated the electrode compartment from an external solution containing 0.1 M KCl, 0.05 M phosphate buffer - pH 7.0, 0.01 M KNO_3, and 0.006 M $MgSO_4$. The solution was equilibrated with 1% oxygen, 5% CO_2 in nitrogen and kept at 22-25 degrees C.

Interference filters (peak transmission 80%, bandwidths 15 and 30 mμ at 40% and 1% transmission, respectively) and heat-absorbing filters isolated the monochromatic beams from 1000 watt tungsten lamps. Identical, coincident, homogeneous beams fell either on the electrode or on a thermopile.

After mounting on the electrode, cells were equilibrated and

illuminated for 2-3 hours. Subsequently, for enhancement measurements, illumination was given in the sequence red, dark, blue, blue plus red, blue, dark, red, dark. Periods of 4-8 minutes insured attainment of 97% of steady state rate.

Like Myers and Graham (1), saturated rates of photosynthesis of 20-60 μl O_2 μl^{-1} cells hour^{-1} were found. Respiratory rates, estimated as described elsewhere (2), were about 1 μl O_2 μl^{-1} cells hour^{-1}.

OBSERVED FUNCTIONS

Due to the length of the measurements, the functions $E(I_1, I_2)$ and $D(I_1, I_2)$ had to be characterized from the results of experiments, each limited to 1, 2, or 3 planes intersecting the enhancement surfaces. Fortunately, the consistency among a number of experiments permitted establishing the semi-quantitative character of the E and D surfaces with no more than modest quantitative errors.

Figs. 1 and 2 show the two surfaces obtained. Fig. 1 shows that E increased with blue illumination (far-red being constant), reached a maximum for $p_1/p_2 \sim 5$, and declined slowly with further increases in p_1. With blue constant, E declined from a maximum

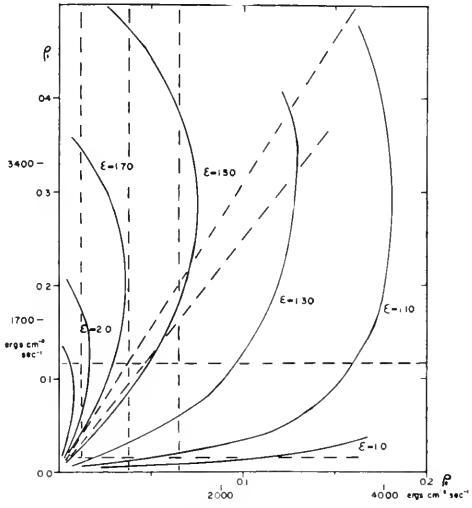

Fig. 1. $E(p_1, p_2)$. Contours of E estimated from experimentally determined profiles (dashed lines).

value as far-red illumination increased. Maximum enhancements were limited to a small region of very weak far-red ($p_2 \sim 0.005$ to 0.010) and blue illumination such that $p_1 \sim 0.05$ to 0.15.

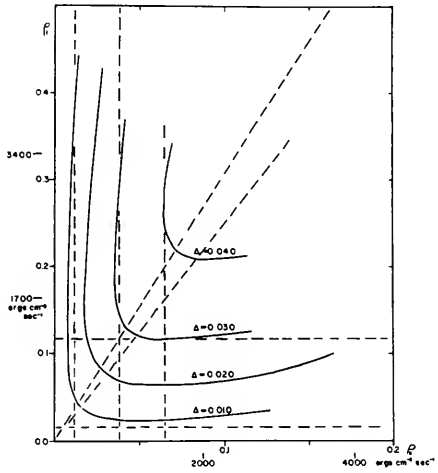

Fig. 2. $D(p_1, p_2)$. Contours of D estimated from experimentally determined profiles.

Fig. 2 shows that D depends on blue and far-red in qualitatively similar ways. As one illumination increases, the other being constant, D rises sharply, then more gradually, then becomes constant. At very high illumination, D apparently decreases. When both illuminations increased in a fixed ratio, D continued to increase -- no maximum being reached with the illuminations employed. The highest values of D obtained were ~ 0.04 and occurred with $p_1 = 0.2$ to 0.3 and $p_2 = 0.1$.

PREDICTED FUNCTIONS

Both the "spill-over" and "separately packaged pigment" models assume that the rate of photosynthesis is limited to the rate of the slower of two required photoreactions. (We assume here that both photoreactions must proceed at the same rate as the overall rate of photosynthesis.) In both models, absorbed far-red quanta are presumed to be distributed in a fixed ratio between far-red and short-wave photoreactions, distribution to the far-red reaction predominating. Accordingly, the rate (p_2) of photosynthesis in far-red is limited to the rate of the short-wave reaction. Then ...

$$p_2 = ai_2 \tag{3}$$

where i_2 is the rate of absorption of far-red quanta and a is the fixed fraction ($< 1/2$) of absorbed quanta acting in the short-wave reaction. Of the rate $(1-a)i_2$ of absorption of far-red quanta by the far-red system, $(1-2a)i_2$ is in excess of the rate of sensitization of the short-wave reaction, and is, therefore, wasted unless balanced by short-wave illumination.

Spill-over Model. According to this model, short-wave quanta are absorbed predominantly by pigments directly associated with the short-wave reaction, but the excitation energy can be transferred to the long-wave reaction when this reaction tends to be the slower. Thus short-wave quanta are presumably distributed in a flexible manner tending to equalize the rates of both reactions. In short-wave light alone, there would be a perfectly balanced distribution, half the absorbed quanta going to each reaction. Then

$$p_1 = i_1/2 \tag{4}$$

where p_1 is the rate of photosynthesis and i_1 is the rate of absorption of short-wave quanta.

In short-wave and far-red lights together, absorbed short-wave quanta go predominantly to the short-wave reaction tending to balance the predominant utilization of far-red quanta in the far-red reaction. In far-red and limiting short-wave illuminations, the rate (p_{12}) will remain limited to the rate of the short-wave reaction:

$$p_{12} = ai_2+bi_1 \tag{5}$$

Here, b ($> 1/2$) is the maximum fraction of absorbed short-wave quanta which can be utilized in the short-wave system -- i.e., (1-b) of short-wave quanta are constrained to act in the far-red system.

As short-wave illumination increases, a point is reached where the rates of introduction of quanta into the short-wave and far-red systems are equal, and both rates equal one-half the total rate of absorption of quanta:

$$p_{12} = (i_1+i_2)/2 = ai_2+bi_1 = (1-a)i_2+(1-b)i_1 \tag{6}$$

At this point no far-red quanta are wasted and any additional

increment of short-wave illumination will be divided equally between both reactions. Hence, at this balance point, and for all higher short-wave illuminations, E and D have fixed maximum values.

From equations 3 through 6, expressions for E and D are easily obtained, either in terms of i_1 and i_2, or p_1 and p_2. Thus, for i_1 less than balancing

$$E = 1+(b-\tfrac{1}{2})i_1/ai_2 = 1+(2b-1)p_1/p_2 \tag{7}$$

$$D = (b-\tfrac{1}{2})i_1 = (2b-1)p_1 \tag{8}$$

and, for just balancing and higher short-wave illuminations

$$E = 1/2a \tag{9}$$

$$D = (1/2a - 1)p_2 \tag{10}$$

Also, from equation 6, is obtained an expression for the ratio p_1/p_2 at the balance point

$$p_1/p_2 = \frac{2a-1}{2a(1-2b)} \tag{11}$$

Separately packaged pigment model. Here, no energy transfer is assumed possible between the two systems. As a result, absorbed short-wave quanta, like far-red quanta, are presumed to be divided in a fixed ratio between the two photoreactions, the preponderance of short-wave quanta acting in the short-wave system. Then the rate of photosynthesis in short-wave light is limited to the rate of the far-red reaction, giving in place of equation 4,

$$p_1 = (1-b)i_1 \tag{4a}$$

Again, simultaneous short-wave and far-red illuminations permit a balancing of rates, and equations 5 and 6 give the rate p_{12} for less and more than balancing short-wave illumination. Then, from equations 3, 4a, 5, and 6 expressions for E and D are derived

$$E = 1+(2b-1)i_1/ai_2 = 1+(2b-1)p_1/(1-b)p_2 \tag{7a}$$

$$D = (2b-1)i_1 = (2b-1)p_1/(1-b) \tag{8a}$$

$$E = (1/a)-1 \tag{9a}$$

$$D = \left(\frac{1}{a}-2\right)/p_2 \tag{10a}$$

$$p_1/p_2 = \frac{1-b}{(2b-1)}\left(\frac{1-2a}{a}\right) \tag{11a}$$

Comparison of models. From experimental values for maximum enhancement and for the ratio p_1/p_2 needed to obtain maximum enhancement, the values of a and b can be calculated from the equations 9 and 9a and 11 and 11a, respectively. From our experimental values $E_{max} \sim 2.5$ and $p_1/p_2 \sim 5$, a = 0.20 and b = 0.65 for the spill-over model, and a = 0.29 and b = 0.57 for the separate package hypothesis.

When these values for a and b are inserted into equations 7 through 10, or into equations 7a through 10a, one obtains, for short-wave light limiting,

$$E = 1+0.30\ p_1/p_2 \tag{12}$$

$$D = 0.30\ p_1 \tag{13}$$

and, for short-wave light greater than balancing,

$$E = 2.5 \tag{14}$$

$$D = 1.5\ p_2 \tag{15}$$

Thus, both models, fitted to the same experimental data, lead to identical numerical equations for E and D as functions of illumination.

COMPARISON OF DERIVED AND EXPERIMENTAL FUNCTIONS

The predicted functions E and D, given for both models by equations (12) through (15), are shown in Fig. 3. Comparison with the experimental functions (Figs. 1 and 2) shows that, for small p_1 and p_2, the predicted and experimental surfaces are in good agreement, both with respect to the general shape of the contours as well as to the positions of contours of a given value. Despite these points of similarity, there are, however, two important discrepancies.

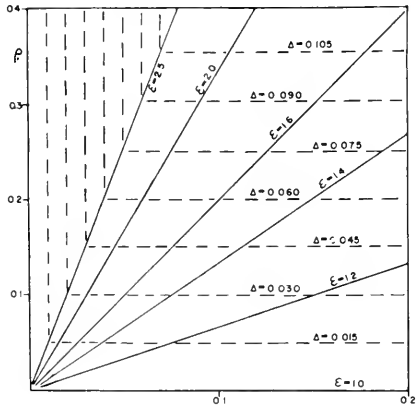

Fig. 3. $E(p_1, p_2)$ and $D(p_1, p_2)$. Predicted by spill-over model (a = 0.2, b = 0.65) and separate package model (a = 0.29, b = 0.57). Everywhere to the left of the contour E = 2.5, E maintains its maximum value of 2.5

Discontinuity of Derived Function. One discrepancy is the discontinuity of the predicted E and D functions along the contour line E = 2.5. In the spill-over model, this discontinuity reflects the assumption that, with p_1 smaller than balancing, short-wave quanta are divided in the ratio b/(1-b) between the two photoreactions, while with p_1 greater than balancing, the short-wave quanta in excess of those needed to reach the balance are divided equally between the reactions. To remove this discontinuity, one could assume that the ratio of division of short-wave quanta changes in a continuous fashion as p_1/p_2 increases. Offhand, such a dependence would seem a conceivable property of an energy-transfer mechanism.

For the separate package hypothesis, the discontinuity is more difficult to explain, since, in this case, b and a represent simply the fixed fractions of absorption by the two separate pigment systems. Possibly, a plausible explanation could be found in terms of the kinetics of dark reactions associated with the photoreactions.

Saturation Effect. One notes a second discrepancy in that, whereas the contours of E of the derived function are everywhere straight lines radiating from the origin, the contours of the experimental function are straight only for low p_1 and p_2, and, for higher p_1 and p_2, curve away in a counterclockwise sense.

We suggest that this curvature of the contours is associated with a curvature of the illumination curve of photosynthesis -- i.e., with the approach to saturation.

The suggested role of saturation is based on an analysis of which only the main points can be given here. In this analysis, it was assumed that the spill-over and separate package models -- and in particular the rate equations 3 through 6 -- refer to the photochemical production of an intermediate which is subsequently converted into products by a dark reaction, which is limiting in high illuminations. In this case, the observed rates of photosynthesis (P_1, P_2, and P_{12}) are non-linear, saturable functions of the light-proportional rates (p_1, p_2, and p_{12}) of intermediate formation. The analysis showed that only a very slight curvature of the illumination curves $P_1(I)$ and $P_2(I)$ is necessary to explain the observed curvatures of the E and D functions. Specifically, illumination curves which would deviate on the average, by only 0.2% of saturating rate from a straight line (over the range from 0 to 1/3 of saturating rate) suffice to explain the observed E and D functions. This small curvature of the illumination curves could exist within the experimental error associated with our apparently linear illumination curves.

CONCLUSIONS

The following results of our work would seem to be of some interest. To begin with, our results appear to be the first to establish the dependence of enhancement over a fairly wide range of both short-wave and far-red illuminations. By expressing this dependence in terms of E and D, functions of the rates (p_1, p_2) relative to the rate of saturated photosynthesis, enhancement can be unambiguously referred to ordinary illumination curves of photosynthesis. This point is noteworthy because many earlier works have given ratios, such as E, without statement of rates; in such cases, it is impossible to assess the importance of respiratory and saturation effects. Our results emphasize also that E and D are very different functions; E can be maximum only at very low far-red illumination while D increases steadily up to comparatively high illuminations.

Secondly, our analytical formulations of the spill-over and separate package models give a theoretical basis for quantitative description of the parameters governing enhancement. Thus, the coefficients a and b are the wavelength-dependent parameters of enhancement which one should seek from action spectra. Unhappily, both models lead to equivalent illumination dependences of

enhancement. However, as Myers (3) has pointed out to us, different maximum limits to enhancement are predicted by the two models. Thus, for the spill-over model, the ratio of $(p_{12}-p_1)/p_2$ could not exceed 2.0 (assuming equal rates of both photoreactions), whereas higher values would occur with the separate package hypothesis; thus, the formulations promise to be helpful in discriminating experimentally between the two models.

Thirdly, the formulations give a ready explanation to some previous observations. For example, the increase in maximum E, and the increase in the ratio p_1/p_2 required to obtain maximum E, which Myers and Graham (4) reported to attend increasing wavelength of far-red, are predicted (equations (9) and (11), or (9a) and (11a)), assuming a declines with increasing wavelength. Myers and Graham also found that the slopes of curves representing $E(p_1)$, for p_2 constant, in the region of small p_1, were independent of far-red wavelength. This agrees with equations (7) and (7a), which show the slope depends only on b.

Finally, our analyses suggest that, aside from the discontinuity discussed above, the general semiquantitative dependence of enhancement on illuminations can be explained on the basis of either model, provided saturation is taken into account. Conversely, our findings indicate that saturation must generally be reckoned with, even at low illuminations normally regarded as within the linear portion of an illumination curve. For example, in determining a maximum value of E, care must be taken that p_1 not be much in excess of that value just giving maximum E; otherwise a less than maximum value of E will be obtained.

This work was supported by the National Science Foundation.

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THE RELATION BETWEEN PIGMENT CONCENTRATION AND PHOTOSYNTHETIC CAPACITY IN A MUTANT OF CHLAMYDOMONAS REINHARDI [1]

G. C. McLeod[2], G. A. Hudock[3], and R. P. Levine

The wild type strain, 137 c, of Chlamydomonas reinhardi is capable of phototrophic growth in the light in a minimal medium with carbon dioxide as a sole carbon source (1). It can be grown in the dark if the minimal medium is supplemented with 0.2 percent sodium acetate. Both light and dark grown cells have a chloroplast and photosynthetic pigments (2,3).

In contrast to the wild type there are several mutant strains (2,4) denoted as yellow-in-the-dark or y strains which are unable to form a chloroplast or synthesize chlorophyll in the dark. Growth in the dark results in a two-fold decrease in chlorophyll content with each cell division. The most striking feature of the dark grown yellow cells is the absence of a lamellar structure of the chloroplast (5).

When the dark grown yellow cells are placed in the light chlorophyll synthesis precedes lamellar formation. A normal chlorophyll content of 1.5 to 2.0 ug/10^6 cells is attained in 8-10 hours. During this exposure to light the doubling time of the chlorophyll is 80-90 minutes whereas the doubling time of the cells is 10 hours. The presence of the carotenoid pigments is not sufficient for lamellar formation (6). The first detectable lamellae are found after two hours of illumination by which time the chlorophyll content is between 0.5 to 0.7 ug/10^6 cells or 30 percent of the normal complement. At the end of the regreening period the chlorophyll content and chloroplast structure of the yellow cells are indistinguishable from those of the wild type strain. Thus, the y-2 strain of C. reinhardi during bleaching and regreening provides a biological system suited for the study of the development of photosynthetic capacity under diverse pigment compositions.

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Several photochemical reactions take part in the formation of the chloroplast pigments. A lag period is followed by a period of pigment synthesis. (Figure 1)

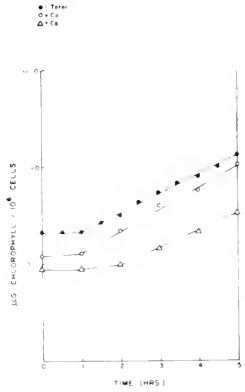

Figure 1. Synthesis of total chlorophyll, chlorophyll a and chlorophyll b in regreening cultures of dark grown y-2.

Little is known about the lag period in chlorophyll formation [6]. Its existence probably indicates that there are certain processes required to initiate a further production of protochlorophyll in the cell [7]. Another change, possibly taking place during the lag phase, is an increased energy transfer from the carotenoid pigments to chlorophyll [8]. Our experiments show that the lag period is not influenced by a variation of the intensity or the wavelength of illumination. Following the lag period, the light intensity controls chlorophyll synthesis. The minimal light intensity for chlorophyll synthesis is between 10 and 20 foot candles. Repeated measurements show the rate of chlorophyll synthesis increases almost linearly with an increase in light intensity above 20-50 foot candles, reaches a maximum rate at 2500 foot candles, and, thereafter, decreases as the light intensity increases.

The ratio of chlorophyll a to chlorophyll b is constantly changing during regreening. At the start of regreening the ratio is 1.3, increases 2.4 at a total chlorophyll level of 0.5 to 0.7 ug/10^6 cells, and reaches 3.0 at a chlorophyll level of 1.30 ug/10^6. (Figure 2)

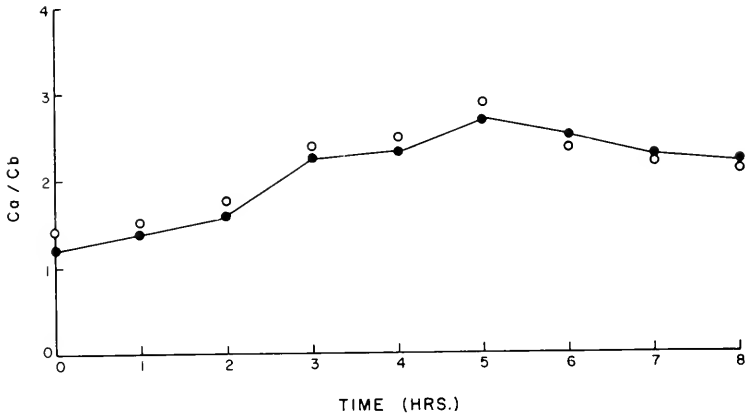

Figure 2. Ratio of chlorophyll a to chlorophyll b (Cal Cb) during the first five hours of exposure of a culture of y-2 to the light following 96 hours of growth in the dark.

The action spectrum for the formation of chlorophyll during regreening was measured by illuminating cultures of dark grown yellow cells with wavelengths of monochromatic light-half band width 5-7 mu- obtained with Bausch and Lomb interference filters backed with Corning Glass filters. The intensity of the monochromatic light for the regreening experiments was always below the level of saturation obtained with white light. The chlorophyll formed was extracted and measured spectrophotometrically (9,10). The effectiveness of the monochromatic light in chlorophyll accumulation was calculated as the reciprocal of the relative number of quanta absorbed in different parts of the spectrum to produce the same quantity of chlorophyll as produced with 650 mu illumination.

The action spectrum of a 96 hour dark grown yellow cells shows maxima at 420-445 mu, 650 mu, and 594 mu, and the relative efficiencies are in this order. (Figure 3) There is a suggestion of a shoulder at 680 to 690 mu. This spectrum agrees with the action spectrum of "greening" in etiolated Avena seedlings, and with that of bleached Euglena cells (11,12); and, the absorption spectrum of protochlorophyll. The action spectrum was also measured at different chlorophyll levels. All procedures revealed that there is a shift in the red portion of the spectrum during chlorophyll synthesis.

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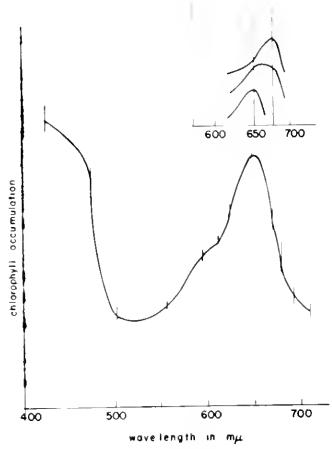

Figure 3. Action spectrum of "greening" of a 96 hour dark grown culture of y-2. The insert shows the shift of the red peak of the action spectrum during chlorophyll synthesis.

At a chlorophyll level of 0.5 to 0.7 ug/10^6 cells the 650 mu peak broadens to 680-690 mu. Above a chlorophyll level of 0.7 ug/10^6 cells there is a shoulder at 650 mu and the major peak at 684 mu. The measurements do not show a shift in a corresponding peak in the blue portion of the spectrum.

Below chlorophyll levels of 0.5 ug/10^6 cells the peaks in the action spectrum are probably due to the absorption of protochlorophyll. The broadening of the peak with further chlorophyll accumulation indicates an increasing participation of chlorophyll a. It is fully active when the peak shifts to 680 mu as the level of pigment approaches that of the light grown cells.

If a photosynthetic reaction is directly limited by the chlorophyll content it should decrease at the same rate as chlorophyll content in the dark grown y-2. On return to the light, the rate of such a reaction would not be expected to increase until after chlorophyll synthesis has begun. On the other hand, the rate of a photosynthetic reaction which is not limited by the chlorophyll content, but by other coincidental factors, will decrease at a different rate.

A test of this hypothesis is to examine carbon dioxide fixation and oxygen evolution during bleaching and regreening. The rate of carbon

dioxide fixation decreases in a manner almost identical to that of the chlorophyll content during growth in the dark. On return to the light, there is a lag period similar in length to that observed for chlorophyll synthesis and then a rapid rise in the rate of carbon dioxide fixation which is followed by a return to a steady state level comparable to that of the light grown cells [3]. The decay of the carbon dioxide fixation during the light period indicates an increased respiration during the synthesis of chlorophyll.

A plot of the oxygen evolution against chlorophyll content during bleaching and regreening is given in Figure 4. In the dark, after a lag period of one generation, oxygen evolution decreases two-fold with each cell division. In the light, following a lag period, there is a small but recurring oxygen evolution similar to that reported by Fork [13]. Above chlorophyll levels of 0.5 to 0.7 ug/10^6 cells, there is a rapid increase in the rate of oxygen evolution. The maximum rate is not attained until the normal chlorophyll complement of 1.5 to 2.0 ug/10^6 cells is restored.

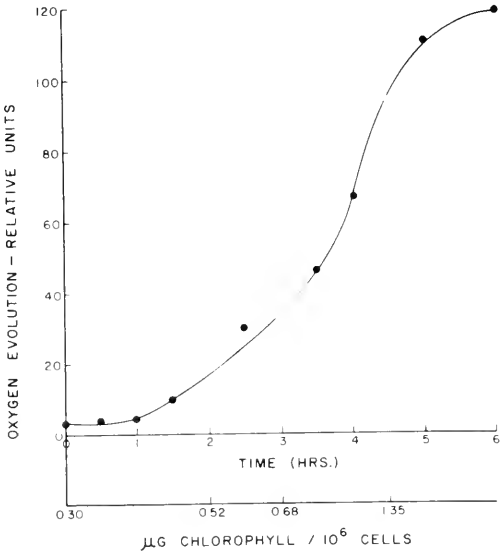

Figure 4. Development of oxygen evolution in a regreening culture of dark-grown y-2. Oxygen evolution in relative units is plotted as a function of both time and chlorophyll content.

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During regreening the saturation rate for photosynthesis increases until the ratio of chlorophyll b to chlorophyll a reaches its normal value. After this, the saturation rate remains relatively constant and the highest rate of oxygen evolution is around 680 mu which agrees with photosynthetic action spectra reported by other workers [14] for normal Chlamydomonas. (Figure 5)

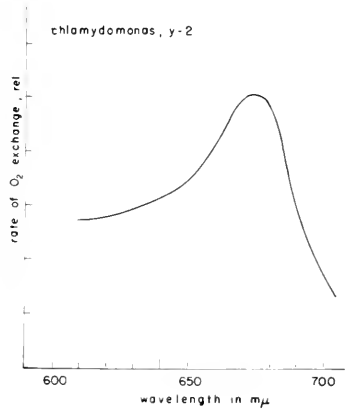

Figure 5. Action spectrum for photosynthesis for a regreened culture of the mutant y-2.

Results from many laboratories [15] emphasize that photosynthesis involves two different photochemical reactions driven by different pigments. Only when both reactions are proceeding in the correct proportions is the maximum rate of photosynthesis attained. Measurements of the enhancement produced by different combinations of wavelengths against pigment concentrations shows that it varies from 1.0 to 1.74 as the total chlorophyll content goes from 0.5 to 1.3 ug/10^6 cells and the Ca/Cb ratio from 2.4 to 3.0. (Figure 6) The absence of an enhancement of photosynthesis below 0.5 ug/10^6 cells may be interpreted as the failure of pigment interaction. More detailed studies of the effects of wavelengths of light on the partial reactions of photosynthesis are necessary to clarify the specific roles of the different pigments during regreening.

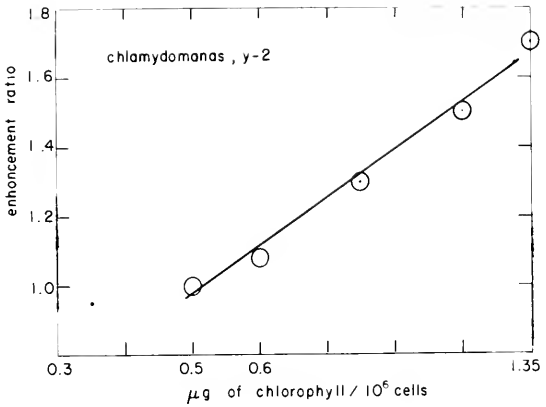

Figure 6. Development of the enhancement of photosynthesis produced by 650-680 mu against chlorophyll content.

The regreening process offers a unique opportunity to study the dependence of photosynthetic capacity on chlorophyll synthesis and other coincidental factors. The kinetics of the decrease of the rate of CO_2 fixation in preparations of dark grown cells, and the kinetics of the increase upon return of the cells to the light is essentially identical to the change in chlorophyll content. It is clear that the fundamental reaction of oxygen evolution begins when only a small amount of chlorophyll is present in the cells, and in the absence of highly organized structural units [16]. The rapid increase in the rate of oxygen evolution at a critical chlorophyll level suggests that either new structures must be built or new components must be formed for the development of an active photosynthesis. These structures could then provide for physical separation of products and even for their transport to regions of the cells suitable for their protection and metabolism. It is certain that the proteins particularly the chloroplastic proteins play a predominant role since the function of the chlorophylls not only depends on the activity of several enzymes, but also on photochemical reactions in which the properties of the pigment protein complex are essential features. Several forms of this complex appears to exist and to be able to change into one another during development. The regreening cell is a dynamic system whose photosynthetic capabilities are bound to the chloroplast (including structural features) and vary with the environmental conditions.

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FOOTNOTES

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V. RESPIRATION, PHOTOSYNTHESIS, AND HYDROGEN METABOLISM

PHOTOREACTIONS AND RESPIRATION

George Hoch and Olga v. H. Owens

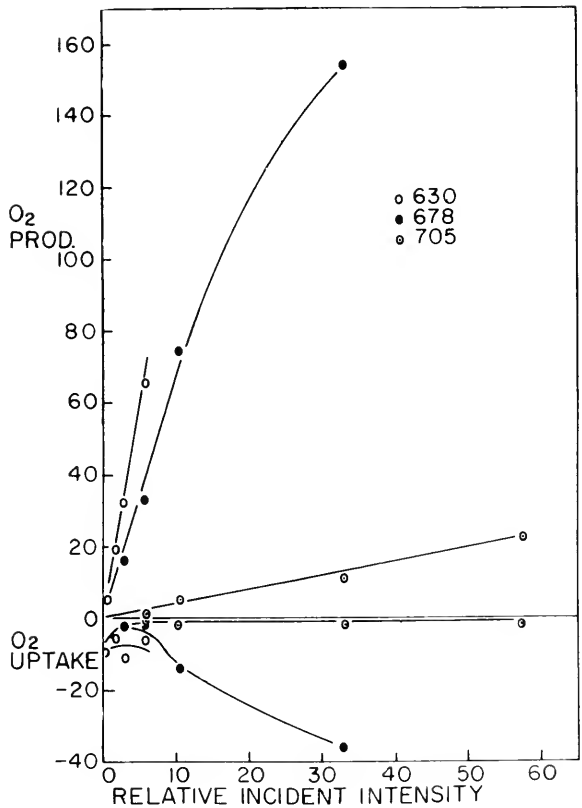
Living cells obtain energy for self-synthesis by three paths, they are: (1) dismutation of chemical compounds; (2) oxidation of chemical compounds; (3) utilization of electromagnetic radiation. This symposium is concerned with the last process, photosynthesis. Photosynthesis is a particularly apt name, but is used too often in a limited sense, that is, the synthesis of sugar or sugar polymers from carbon dioxide and water at the expense of light energy.

The purpose of our investigations was to determine the relationship between two of these energy supply paths, namely oxidation (as evidenced by respiration) and light utilization. The subject has a long and somewhat varied history, ranging from a suppression of respiration in the light to an acceleration of large magnitude. In particular, the effects occurring at low light intensities are interesting, if for no other reason than how they affect measurements of the quantum yield, chromatic transients and Emerson "enhancement".

Following the lead of Allen Brown and coworkers[1,2] we have employed a mass spectrometer in these investigations. The instrument has been described[3] and differs from others in that it directly measures the gas concentrations dissolved in the liquid phase.

Oxygen uptake and production at various wavelengths.

Figure 1 describes the response of oxygen uptake and production to increasing light intensities of three wavelengths in Anacystis nidulans. This figure effectively summarizes our results; although the effects may differ quantitatively from day to day, the qualitative features are consistent[4]. These features are that light given to chlorophyll a suppresses oxygen uptake while light given to the accessory pigment, phycocyanin, increases oxygen uptake. The overall feature in chlorophyll a light is a broken curve when net oxygen exchange is plotted as a function of intensity. This effect was first observed by Kok[5] and has been termed the "Kok effect".



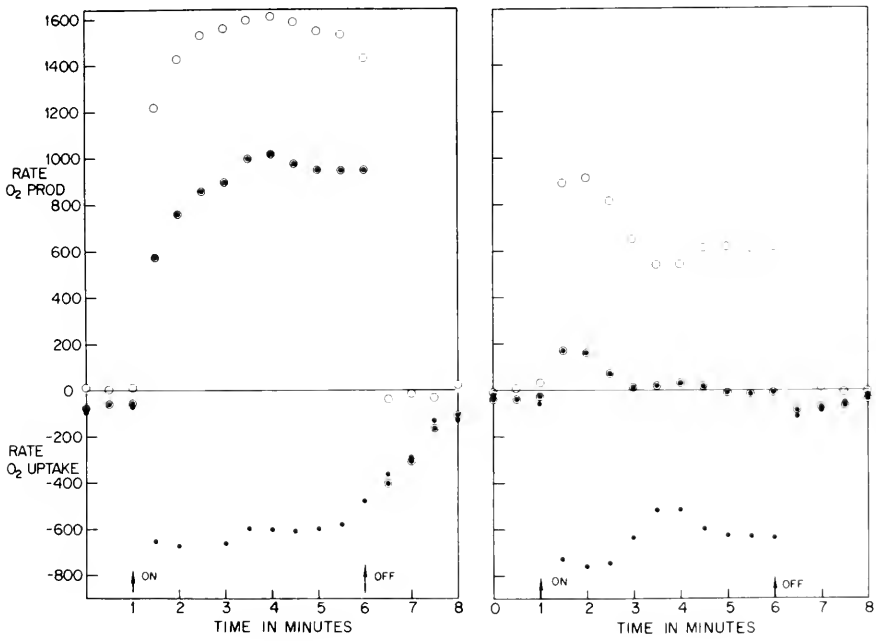
The increased oxygen uptake occurs at short wavelengths in both blue-green and green algae. The magnitude of the increase is variable from one culture to another, doubtless depending on the prior history of the cells. The increased uptake has an irregular response to light intensity, as it generally does not start until sufficient intensity to compensate dark respiration (or somewhat more) has been given. At intensities below this point, oxygen uptake does not change or is slightly inhibited. This increased uptake can also lead to a small break in the net gas exchange curve.

Effect of Cyanide on Oxygen Uptake and Production.

It has been known for some time that the effect of cyanide on respiration and photosynthesis varies with the

organism under study. Warburg reported in 1920 that, even in relatively high concentrations of cyanide, respiration of Chlorella was unaffected. Photosynthesis on the other hand was inhibited by cyanide but only in strong light. Warburg made the observation that cyanide inhibited photosynthesis only to the compensation point. In contrast to Warburg's Chlorella, Gaffron found that a species of Scenedesmus had a respiration which was not only sensitive to cyanide but which was more sensitive than photosynthesis(6)

Figure 2 is an experiment done with saturating light in which oxygen uptake, oxygen production, and net oxygen exchange are shown as functions of time for zero cyanide and 10^{-3}M cyanide in Anacystis nidulans.

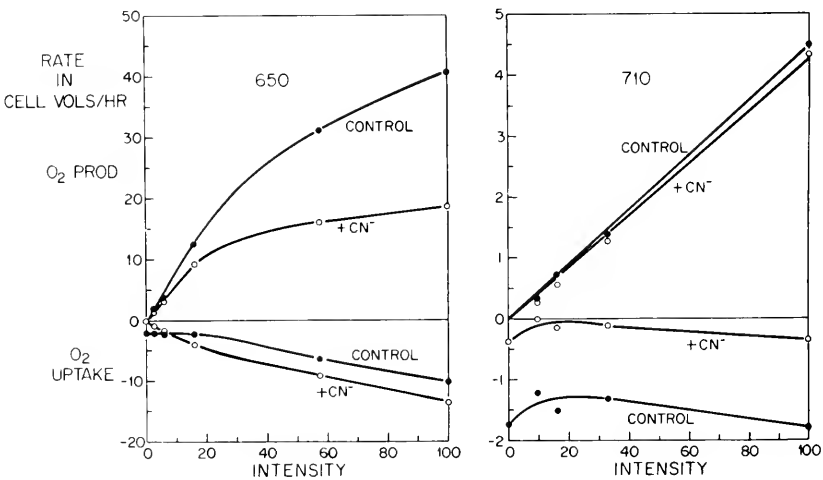


In the control sample the typical increase in oxygen uptake occurs at saturating intensities but it is countered by an even larger oxygen production. The result is a positive net gas exchange. Addition of 10^{-3}M cyanide causes a reduction in the rate of oxygen production without a con-

comitant decrease in oxygen uptake so that the net gas exchange is very much less. Also evident in the figure is that the cyanide effect is somewhat progressive with time in the light. Cyanide at 10^{-3}M, while inhibiting photosynthesis to about compensation, is actually allowing recombination of the photoproducts, so that the light reactions per se are being inhibited only slightly. This perhaps explains the failure of cyanide to cause fluorescence changes similar to other inhibitors of photosynthesis (7).

As the cyanide concentration is increased (to 10^{-1}M) both oxygen production and uptake go to zero. Although the effects of cyanide appear complex the light dependent uptake of oxygen is only indirectly affected by cyanide. The drop in rate of light uptake results from the action of cyanide on the oxygen production mechanism, which in turn brings about a lowered production of the oxidizable reductant.

An investigation was made of the effect of cyanide at lower light intensities at wavelengths where either one or the other of the two photosystems predominates. Figure 3 shows such an experiment with Scenedesmus. The controls



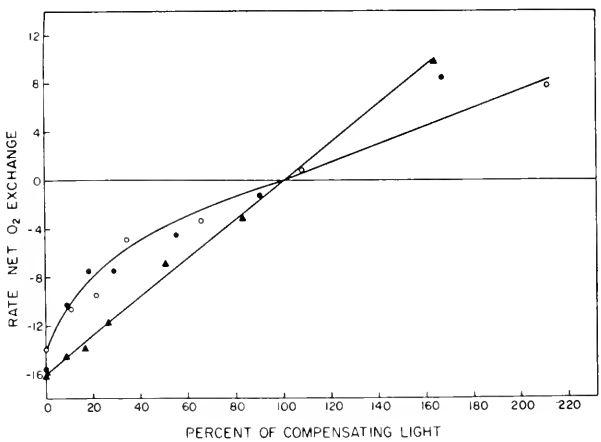
show the typical response which we have found at two wavelengths. When the light is saturating, as shown in the higher intensities of 650 light, the cyanide effect is large, inhibiting photosynthesis to 50%. At lower intensities, on the linear part of the curves, the cyanide effect

is still present, but is considerably smaller, both wavelengths exhibiting a slight but significant inhibition in oxygen production. The results are in general agreement with Warburg in demonstrating that the most pronounced inhibition is observed in saturating light.

Oxygen uptake shows an interesting effect which might help to explain some of the anomalies which we have seen in our experiments and which undoubtedly can lead to discrepancies in data. At this concentration of cyanide (10^{-3}M) dark respiration was inhibited to near zero. With increasing light intensity of 650 mu, respiration increased in a nearly linear fashion until it equalled and then exceeded the uninhibited control. Similar results have been obtained with Anacystis. In no case, however, have we been able to show a significant difference in inhibition of oxygen production with wavelength.

Respiratory effects on measurements of photosynthesis.

The suppression of respiration affects certain measurements of photosynthesis. Figure 4 shows the net oxygen exchange of Anacystis for several wavelengths. The broken



curves for chlorophyll a regions make obvious the reasons why Emerson measured high efficiency for this region while others measured low efficiencies, it all depends where on the light curve the measurements were made. A similar explanation suffices for "negative enhancement" or "de-enhance-

ment". This effect is a general one, Emerson [8] found it in the blue-green Anacystis, the diatom Navicula, and in the red algae Phorphyridium. Govindjee and Rabinowitch[9] showed the effect in Chlorella. The answer to this is again suppression of respiration. The first light beam gives both oxygen production and suppression of uptake while the second yields only photosynthesis, therefore, the second beam gives less net oxygen exchange when added to another beam also absorbed by chlorophyll a than it did when measured by itself. This effect also makes the observed enhancement, in those regions where it occurs, less than the true amount[10]. This is shown in Table 1. The observation of negative enhancement in Chlorella is especially reassuring because here, as we mentioned before, the suppression of uptake is often too small for us to measure with the isotopes.

	Mass Spectrometer				"Man"	Enhancement	
	Prec. Dark Uptake	Light Uptake	Light Prod.	Net Exchange	Prod.	Mass Spec.	"Man"
705	- 30.6	- 20.1	4.4	- 15.7	14.9		
678	- 31.8	- 20.5	91.9	+ 71.4	103.2		
705 + 678	- 34.2	- 23.6	97.9	+ 74.3	108.5	1.4	0.36
705	- 32.7	- 18.0	4.7	- 13.3	19.4		
630	- 30.7	- 27.4	48.6	+ 21.2	51.9		
705 + 630	- 31.3	- 24.7	68.3	+ 43.6	74.9	4.2	1.2

In several experiments we have found positive values for enhancement when we superimposed light beams of 680 and 705 mu and measured oxygen production with isotopes. However, with a total of 22 different experiments we obtained an average enhancement equal to 1.1, well within our experimental error. While we do not feel this disproves the possibility of enhancement between these wavelengths, any enhancement effect must be of small magnitude.

Influence of cyanide on enhancement.

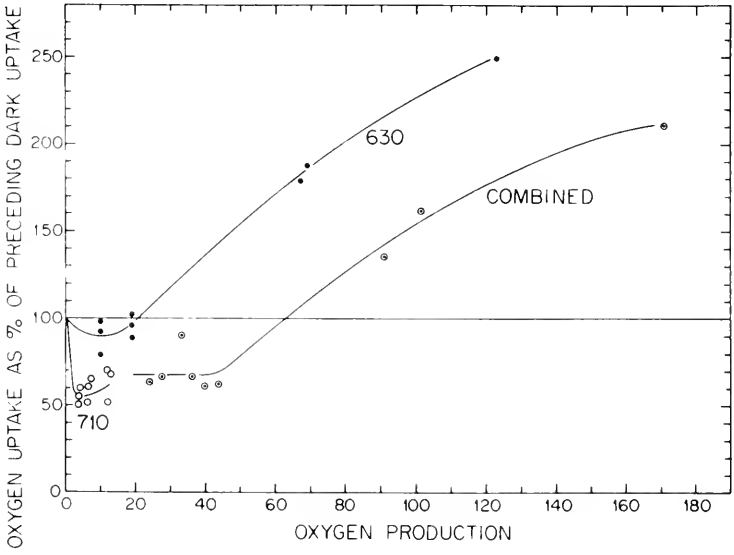
Table 2 shows some rather unexpected results. Although our attempts to show a specific effect of cyanide on one or the other wavelength have not been successful the inhibition does have an effect on enhancement observed with isotopes. Addition of cyanide slightly decreases the oxygen production of both beams (much less, of course, in this

intensity region than it does at saturation) but increases the extra oxygen arising as a result of mixing the beams. This results in an increased enhancement factor.

		Oxygen Production		Δ 710		Enhancement	
		Control	+CN⁻ 10^{-3} M	Control	+CN⁻ 10^{-3} M	Control	+CN⁻ 10^{-3} M
I	710	7	5				
	649	241	187				
	710 + 649	267	223	19	31	3.7	7.2
II	710	10	7				
	649	207	180				
	710 + 649	240	213	23	26	3.3	4.7
III	710	48	38				
	649	34	21				
	710 + 649	86	76	4	17	1.1	1.4

Response of O_2 uptake to mixed beams.

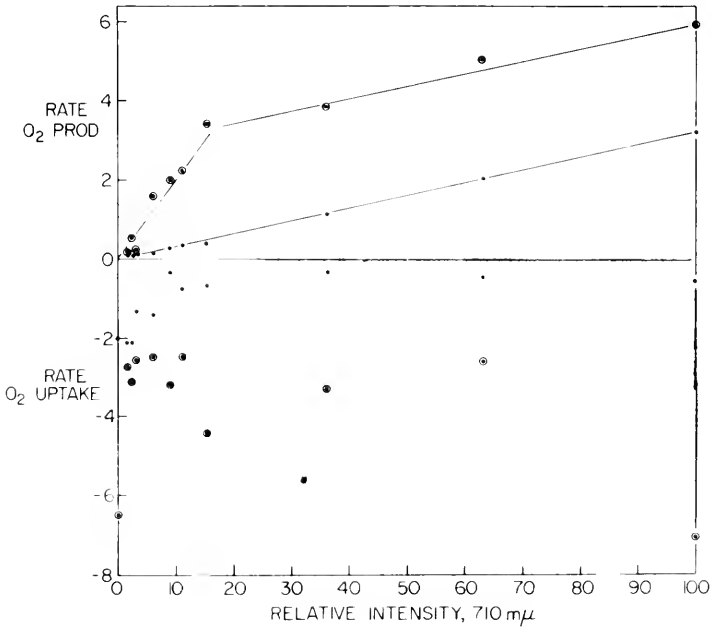
Figure 5 shows the oxygen uptake as a function of oxygen production for 630 mu light, 710 mu light and a combination



of the two beams. At low rates of oxygen production chlorophyll a light causes a depressed oxygen uptake while at these same values oxygen uptake is only slightly inhibited in 630 light. Increasing the rate of O_2 production in accessory pigment illumination leads to an increased rate of uptake above that occurring in the dark.

Combining the beams as shown on the third curve yields an oxygen uptake which, relative to oxygen production, is less than in 630 mu light alone. A combination of beams which increases oxygen production, therefore, reduces oxygen uptake. If oxygen uptake can be considered a "loss" reaction in photosynthesis, it is obviously the long wave photoreaction which assists the short wave reaction. Note also that the inhibition of uptake caused by the far red beam is greater when far red is given to 630 light than when it is added to dark. This is probably simply explained by the fact that more uptake occurs in 630 light than occurs in the dark.

Figure 6 shows an enhancement experiment with Anacystis.



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In this case we consider the response of oxygen production and uptake to increasing intensities of 710 mu light when this light is added to a sample in the dark or to a sample already illuminated with 630 mu light. In the first case oxygen production is a linear function of 710 mu intensity. Oxygen uptake shows the characteristic inhibition at this wavelength. The net gas exchange curve will of course show a break. Now we do the same type of experiment as done by Jones and Myers [11] and put the same 710 mu light on a background of 630. The curve now rises quickly, due, of course, to enhancement, until it has "titrated" the available short wavelength photoproduct and then resumes the slope found for the far red alone. The ratio of slopes in this experiment is 6.5.

Now the crux of the experiment is the response of oxygen uptake during the addition of the 710 mu light to the 630 mu light. The figure shows that in the 630 light alone oxygen uptake is increased and that addition of the 710 mu beam suppresses this uptake. The suppression is essentially complete at the lowest intensity of far red light used. Because of the scatter in the data we obtain in this type of experiment we are unwilling to place much emphasis on the apparent kinetics. Better instrumentation is required to resolve this. Nevertheless, it appears to us that during the intensities in which the far red beam is being enhanced (the steep slope), oxygen uptake is being suppressed. If these results are correct, suppression of uptake is not competitive with enhanced oxygen production, but rather the two can occur simultaneously.

Discussion

The results show that respiratory changes can and do cause many anomalies when the net rate of gas exchange is taken as a measure of photosynthesis. In our experiments we have observed a remarkable linearity of actual oxygen production as a function of light intensity over the lower intensity regions. The respiratory effects are clearly the source of many discrepancies in the measurement of the quantum yield of photosynthesis as a function of wavelength. they also provide explanation for the puzzling phenomen of "negative" enhancement. Perhaps other observations of Emerson such as the extension of the good quantum yields of photosynthesis into the far red by such means as the addition of yeast extract or earth extract or low temperature

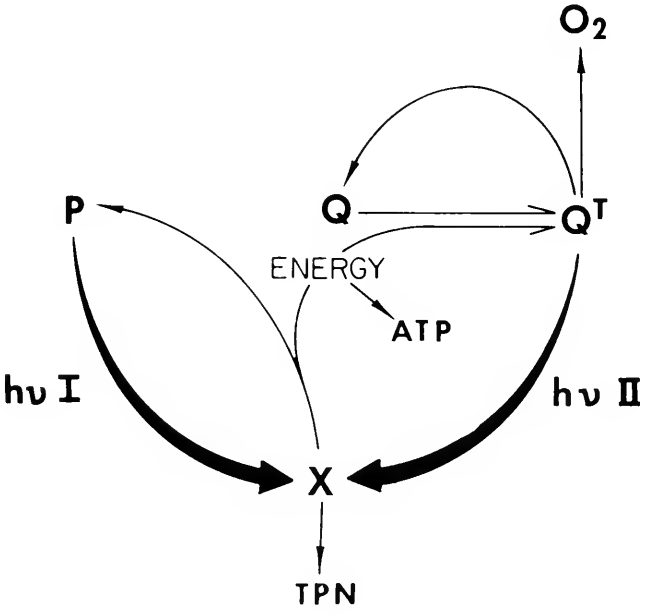
may have also an origin in respiration. The chromatic transients have been ascribed by Blinks to changes in respiration [12]. Above all they indicate that photosynthesis and respiration are not mutally exclusive processes within the cell but rather are interrelated, allowing the cell to function as an entity.

Brown and Weiss [13,14] have found carbon dioxide evolution to be suppressed during illumination of both a green and a brown alga. Furthermore, this suppression was independent of intensity of illumination over a wide range. If this is also the case in Anacystis, which seems likely, then we can assume dark respiration to be inhibited by light. The increased oxygen uptake which occurs when accessory pigments are illuminated may be possibly ascribed to an oxidation of a photosynthetic reductant produced in photosystem II. The mechanism by which chlorophyll a light suppresses respiration is likely to be similar to other systems for control of competing, energy supplying processes. The Pasteur effect has been thought to exert its control over fermentation through the phosphorylating components common to both systems. This may be the case in photosynthesis also. If we were to assume that oxygen uptake is inhibited by the abstraction of reducing equivalents from respiration by an oxidized moiety produced in photosynthesis, then CO_2 evolution would have to continue unabated in the light. It seems more probable that control is exerted through high energy phosphate.

In this regard, it is well to consider the total energy requirements for the growth of micro-organisms or plants at the expense of light energy. Reduction of carbon dioxide to the level of carbohydrate apparently requires two reduced pyridine nucleotides and 3 ATP or 12 PNH and 18 ATP per hexose. From a variety of organisms the ATP demand for synthesis of cell stuff from a supplied hexose is 18 ATP. Hence, per 6 CO_2 fixed the light must supply 12 PNH and 36 ATP or two and 6 respectively per CO_2. Present schemes for photosynthesis (as Hill and Bendall [15] envisage two quanta per electron and that is what is measured for the reduction of TPN in the Hill reaction. However, this supplies only 2 ATP per O_2 instead of 6. The missing 4 ATP could come from a recycling of P700 but at the expense of at least 2 more quanta. Hence, from present data the requirements for growth are 10 hv per CO_2 (uncorrected for respiration). If the lower values found in the literature (as for instance

7.4)[16] are correct either more ATP must be found or the schemes for photosynthesis must be revised.

Since both enhancement effects and respiratory suppression have common sensitizations it is conceivable that they have a common origin. The predominate photoreaction at far red wavelengths is an oxidation of P700 and reduction of a low potential reductant. Recombination of these photoproducts could lead to phosphorylation and therefore inhibition of respiration. It appears some mechanism of this type must operate in the cell to provide growth requirements. The question then, is whether this system is in competition with the oxygen evolution reaction. We would like to suggest that the long wavelength system which we feel provides phosphorylation through recombination is capable of operating independently and is not in competition with oxygen evolution. Rather, it makes some energetic product which assists the oxygen evolution reaction. This product is presumably capable of conversion to ATP. We have tried to outline this in the rather general scheme shown in Figure 7.



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SOME FLAVIN INTERACTIONS WITH GRANA (SEEN IN A DIFFERENT LIGHT)

Birgit Vennesland

There are currently two completely different ways of conceptualizing the mechanism of photosynthesis. One is so familiar to you that I need not summarize it. On the other hand, we have Warburg's view—shared by a minority and unfamiliar to many. I welcome this opportunity to elucidate Warburg's point of view because, in my opinion, his picture of the mechanism of photosynthesis is right.

Warburg's theories are, in a sense, summaries of the results of his experiments. It is impossible to judge the theories without considering the experiments on which they are based. To do this adequately in the allotted time is impossible. The experiments should be considered not only in detail, but in toto; for the interpretation of a set of observations on isolated chloroplasts rests in part on what one has learned from a study of intact Chlorella. I can't take time here to describe all the important experiments that Warburg has done with Chlorella, but I'll compromise with necessity by outlining briefly a few facts which are particularly important as guides to the interpretation of the phenomena we study with isolated green grana.

SOME EXPERIMENTS WITH CHLORELLA

First, let me recall a set of experiments which show that CO_2 is required for the compensation of respiration by light [1]. If the CO_2 produced in respiration is removed rapidly, light cannot compensate respiration. The experiment is done manometrically, with a Chlorella suspension in the main compartment of the Warburg vessel, and base in the side-arm. Under these conditions, the effectiveness of light in counterbalancing respiration depends on the rate of shaking. Rapid shaking ensures rapid removal of CO_2 and leaves the light nothing to work on. As the rate of shaking is decreased, the light becomes increasingly more effective. This experiment shows that light exerts its effect on the endogenous dark-respiration of Chlorella by way of CO_2.

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Another set of important experiments[2] has to do with the activated CO_2 or O_2 precursor—also called the "photolyt"[2-4]. The results of a demonstration experiment, shown to me by Jetschmann in Warburg's Institute in October, 1961, have been graphed in Fig. 1.

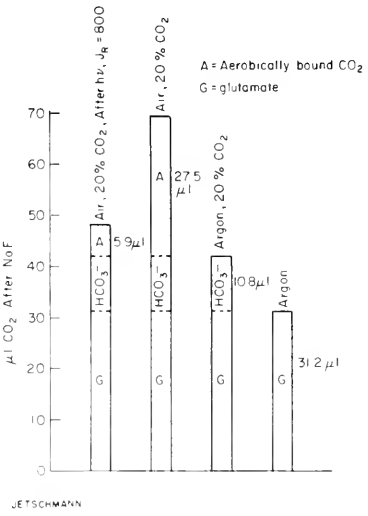

Fig. 1 The Activated CO_2 or O_2 Precursor

Four Warburg vessels are set up with identical contents: 100 µl of Chlorella cells in culture medium. These vessels are gassed with different gas mixtures. The first two vessels contain 20% CO_2/air; the third 20% CO_2/argon, and the fourth, argon. After half an hour of equilibration in the dark, acid fluoride is tipped into the second, third and fourth vessels, but the first vessel is illuminated with a bright light before tipping acid fluoride. The amount of fluoride used is sufficient to inhibit respiration completely. A positive pressure due to release of CO_2 appears in every case after fluoride addition. The bar graph shows the amounts of CO_2 formed when the reactions have gone to completion. The CO_2 released in the fourth vessel is a measure of the amount of glutamate present. This is equivalent to the chlorophyll content of the cells. The CO_2 released in the third vessel gives the glutamate plus the CO_2 bound as bicarbonate when the cells are in 20% CO_2. The CO_2 released in the second vessel is greater than that released in the third vessel by an amount equivalent to the glutamate. This is the aerobically bound CO_2 or the activated CO_2. The first vessel does

not contain much activated CO_2. However, when this vessel was illuminated, there was a pressure burst due to the release of excess O_2. In steady-state photosynthesis, O_2 evolution is accompanied by an equivalent CO_2 consumption, but here O_2 evolution exceeded CO_2 consumption by an amount approximately equivalent to the amount of activated CO_2. In summary, when the cells are incubated in air and CO_2 in the dark, they form a CO_2 compound which is readily converted to O_2 in the light, but which readily decomposes to CO_2 in the dark. Since we don't know the chemical structure of this substance, we represent it by the symbol CO_2^*. Because it is split to O_2 in the light, it has also been called the "photolyt". The conclusive proof of the importance of CO_2^* is the demonstration that it decomposes to O_2 with a quantum requirement of one[4]. The photosynthetic unit is one. One molecule each of chlorophyll, glutamate and CO_2 form a complex which liberates O_2 when it absorbs a photon. The reducing power generated by the light resides in the reduced carbon left behind by O_2 elimination.

It has been claimed that there is no such thing as activated CO_2—experiments with C^{14} are said to reveal no trace of it[5]. In this connection I must emphasize that CO_2^* is consumed as fast as it is formed when the cells are strongly illuminated. Cells performing photosynthesis at maximum rates contain little CO_2^*. If you want to use C^{14} to show that there is no such compound you will of course use a bright light. Cells operating at maximum efficiency must, on the other hand, keep the photolyt reservoir full.

Since there isn't enough energy in one photon of red light to convert one molecule of CO_2 to O_2 and carbohydrate, the energy deficit must be made up. The manner in which this occurs was made clear by the discovery of the light-induced respiration. This phenomenon is quite easy to demonstrate manometrically with Chlorella. Those who wish to see it may be advised not to use too heavy a cell suspension, or most of the cells will be in the dark most of the time, so that most of the light-induced respiration may occur during the illumination period[6].

At this point, it is appropriate to mention the use of labeled oxygen to measure the light-induced respiration. One would expect to see more of this respiration with the O^{18} technique than one can see manometrically. But it is not true that the use of the O^{18} technique will guarantee that one sees all of this light-induced respiration. The method requires a sampling of oxygen from a gas phase, supposedly in equilibrium with the inside of the

cell. Users of this method assume that the isotope ratio in the external gas phase reflects the isotope ratio at the site of O_2 generation and O_2 reutilization inside the cell. This assumption is unjustified. There are bound to be concentration gradients, and there is no way of calculating how large or small these concentration gradients will be.

Personally, I regard the light-induced respiration as a genuine experimental phenomenon. I have seen it. And I think Warburg's experiments prove that the fundamental mechanism of photosynthesis should be represented as an oxygen elimination from a CO_2 derivative, followed by a reoxidation of a part of the reduction product. The stoichiometry of the reoxidation is calculated from the quantum requirement. For an overall quantum requirement of three, 2/3 of the reduced carbon must be reoxidized; an overall quantum requirement of four suggests that 3/4 are reoxidized. When photosynthesis proceeds at low efficiencies, this may be because a larger fraction of the reduction product is reoxidized, or because much of the chlorophyll is not associated with photolyt under the conditions used for the measurements.

All of the preceding points are preliminary to a consideration of some experiments with isolated chloroplasts. In Chicago, we work mainly with chloroplasts from leaves, particularly spinach leaves, and I have described experiments done mainly with Chlorella. This is because it hasn't yet been possible to provide a direct demonstration of the formation and function of CO_2^* in leaves. But it seems reasonable that oxygen-generating photosynthesis should have the same fundamental mechanism wherever it occurs in nature, and we assume this is so. The assumption has received support from the recent demonstration, by Warburg and his associates, that small young lettuce leaves can perform photosynthesis with a quantum requirement not greater than 4.5 per molecule of O_2 released[6].

THE STIMULATION OF THE HILL REACTION BY CO_2

For many years, the occurrence of the Hill reaction in isolated chloroplasts or grana seemed to show that the CO_2-fixing part of photosynthesis could be separated completely from the O_2-evolving part. This is how Hill interpreted his initial observations, and this is how most of you still interpret the Hill reaction. In fact, it is the existence of the Hill reaction with its associated photophosphorylation which provides the best evidence for the statement that the grana function mainly to provide ATP and reducing power

for the Calvin-Benson cycle. The photosynthetic unit of several hundred chlorophyll molecules is regarded as a necessary postulate to provide for the intensification and transfer of the reducing power by way of the catalytic components of the respiratory chain present in the chloroplasts.

If this prevailing view is right, there is no such thing as functional activated CO_2 in the isolated chloroplasts of leaves, and one must conclude that the experiments demonstrating activated CO_2 in Chlorella are incorrectly interpreted, or that Chlorella and leaves do photosynthesis by fundamentally different mechanisms. This is the reason that the demonstrated stimulation of the Hill reaction by CO_2 has such great theoretical importance. It is the fact which reconciles the Hill reaction in grana with the behavior of intact Chlorella[7-10].

We think of the Hill reaction as starting with an elimination of O_2 from CO_2^*, and proceeding with a complete reoxidation of the reduced carbon by the Hill reagent. What we are seeing in the Hill reaction is a modified form of the light-induced respiration. This back reaction is going too far, however. The reduced carbon is reoxidized completely. The mechanism for conserving a portion of the reduced carbon is not operating in the grana.

Of the various naturally occurring Hill reagents, TPN is certainly one of the more interesting. Both in Warburg's view and in Arnon's view, the pyridine nucleotide plays an important role. But the sequence of events is pictured quite differently. Arnon and many others think of the TPN as the reagent which is primarily responsible for transmitting reducing power from grana to the carbon substrates, specifically by way of the triose-phosphate dehydrogenase reaction. Warburg thinks of the reduced carbon (left behind by O_2 elimination) as the primary photo product, which then can reduce TPN to TPNH. As evidence for this view I submit the effect of CO_2 on TPN photoreduction (Fig. 2) [9, 10].

The effect of CO_2 on O_2 evolution with TPN is not difficult to demonstrate. Even intact spinach chloroplasts may show a stimulatory effect of CO_2 amounting to 20%, after a preliminary incubation of one hour. Fig. 2 (left) shows the effect obtained by using broken chloroplasts without a prolonged depletion treatment. A better material demonstrating the CO_2 effect on TPN photoreduction is sonerated Chlorella[9]. Fig. 2 (right) shows two sets of measurements which I made in Berlin with this material. The lower part of the graph shows a repetition of an experiment which Warburg and

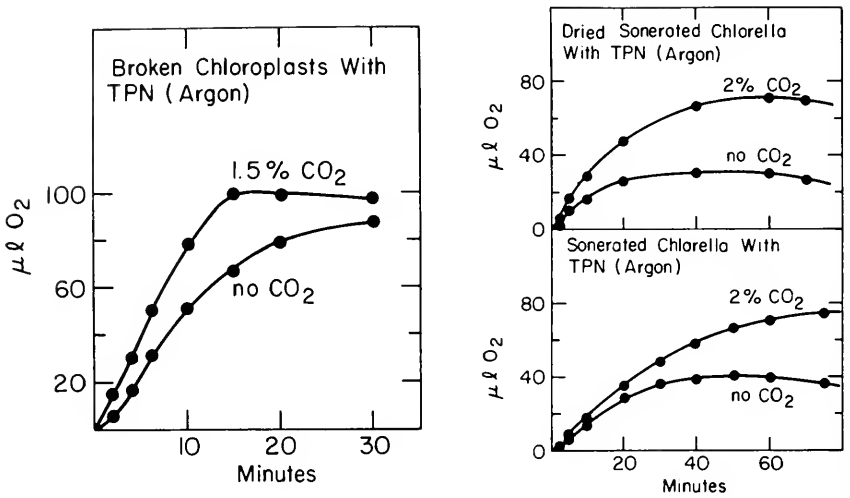

Fig. 2 Effect of CO_2 on Photoreduction of TPN

Krippahl have recently published[9]. The upper part of the graph shows a similar set of measurements made with sonerated Chlorella extract which had first been lyophilized. Sonerated Chlorella extract contains all of the proteins necessary for TPN photoreduction. Since the necessary soluble components have not been removed, it isn't necessary to add them. It is also unnecessary to use any special procedures to remove the CO_2.

The question of the energetics of TPN reduction is an interesting one. There is insufficient energy in one photon of red light to effect a reduction of two molecules of TPN with release of one molecule of O_2. If the reduced carbon generated by O_2 elimination is completely reoxidized by TPN, then some of this reduced TPN must be reoxidized by part of the O_2 in order to achieve an adequate energy balance for the net reduction. With sonerated Chlorella extracts, Warburg and Krippahl have demonstrated that there is a re-reduction of O_2 occurring during the Hill reaction with TPN. Addition of HCN to inhibit catalase results in an inhibition of O_2 evolution, and an accumulation of H_2O_2[9]. There is an apparent shift from an ordinary Hill reaction to a dismutation: $TPN + 2\ H_2O \longrightarrow TPNH_2 + H_2O_2$ This dismutation occurs under an atmosphere of argon. The O_2 necessary for H_2O_2 formation must come from the O_2 evolved in the initial Hill

reaction. But it is impossible to inhibit the H_2O_2 formation by absorbing O_2 out of the gas phase in contact with the illuminated solution. The O_2 is reduced to H_2O_2 before it gets into the gas phase.

I refer you to the paper of Warburg and Krippahl for further details about this interesting reaction. What I want to emphasize here is the fact that this particular system provides a convincing demonstration that oxygen can be formed and reutilized in the liquid phase without entering the gas phase which is supposedly "in equilibrium" with the liquid.

CO_2 EFFECT ON H_2O_2 FORMATION

The formation of large amounts of H_2O_2 by illuminated Chlorella poisoned with HCN was first reported from Warburg's Institute in 1925 by Tanaka. The emphasis in this early paper was on the fact that H_2O_2 is not formed during dark respiration. Now we are toying with the idea that the light-induced respiration—the back reaction——of photosynthesis is at least in part a peroxide respiration.

H_2O_2 formation isn't limited to intact Chlorella. I have been able to demonstrate H_2O_2 formation also by cyanide-poisoned intact leaves. Light is required, just as for Chlorella. With leaves, the first and most obvious effect of light in the presence of HCN is a bleaching of the green pigment. The leaf turns white in red light of an intensity quite insufficient to do damage in the absence of HCN. As the bleaching occurs, a small amount of H_2O_2 (0.1 to 0.5 μmole per 100 mg fresh weight spinach) appears in the water in which the leaf is suspended. One is inclined to regard the bleaching as the consequence of the internally generated H_2O_2. The peroxide that escapes into the medium is only the fraction that hasn't acted chemically to cause bleaching.

Hydrogen peroxide formation by isolated chloroplasts, first reported by Mehler, has been studied extensively and shown to be the consequence of the reoxidation by O_2 of an autoxidizable Hill reagent. There are a variety of naturally occurring substances which may function in this way, and the specific identity of the responsible reagent has to be determined in each case.

The formation of H_2O_2 by intact Chlorella (measured in the presence of 0.01 M HCN) has been shown to be enhanced by CO_2[11]. H_2O_2 formation

by sonerated Chlorella extracts shows a similar stimulation by CO_2, as illustrated in Table I. Sonerated Chlorella extract was incubated in the light

TABLE I

Effect of CO_2 on H_2O_2 Production

Sonerated Chlorella extract (200 µl cells/ml) as indicated, 300 µmoles phosphate, pH 6.6, 50 µmoles KCl, 3 µmoles NaN_3, in volume of 3 ml; air; I_R = 600 µl/min, 20°, 70 min.

Amount extract	CO_2	O_2	H_2O_2
ml	%	µmoles	µmoles
1.0	2	-6.8	9.9
	0	-4.8	5.2
0.25	2	-2.4	3.4
	0	-1.9	1.7

with phosphate buffer, KCl and 10^{-3} M NaN_3 to inhibit catalase. The Table shows the O_2 consumption and H_2O_2 production in the presence and absence of externally added CO_2. If nothing else is happening, one expects two molecules of H_2O_2 to accumulate for each mole of O_2 taken up. This ratio is not observed here, partly because there is a consumption of O_2 in the dark, for which we have made no correction. The CO_2 effect is reflected very clearly in the H_2O_2 formation. There is twice as much H_2O_2 formed in the presence of externally added CO_2 as in its absence.

With spinach chloroplasts, it is more difficult to show the CO_2 effect on H_2O_2 production, but it is there and can be demonstrated after depletion procedures. Here again, H_2O_2 production gives a more sensitive response to CO_2 than does oxygen consumption. With broken chloroplasts, I have obtained 20% stimulation of H_2O_2 production by CO_2 under conditions where the oxygen consumption was apparently unaffected.

THE HILL REACTION WITH FMN

May I turn now to a more detailed consideration of the Hill reaction with FMN. The previous discussion provides a background for the

interpretation of the flavin reaction, which we make as shown in the following equations, in which F represents flavin, and E represents energy.

$$CO_2^* + H_2O \longrightarrow [CH_2O] + O_2 \quad (1)$$

$$2\,F + [CH_2O] + H_2O \longrightarrow 2\,FH_2 + CO_2 \quad (2)$$

$$FH_2 + 1/2\,O_2 \longrightarrow F + H_2O + E \quad (3)$$

$$CO_2 + E \longrightarrow CO_2^* \quad (4)$$

$$F + H_2O \longrightarrow FH_2 + 1/2\,O_2 \quad (5)$$

The photochemistry starts with reaction 1. This is followed by the reduction of flavin as shown in reaction 2. But there is not enough energy in one photon of red light to support a net reduction of two molecules of flavin with evolution of one molecule of O_2. To make up the deficit we postulate that half of the reduced flavin is reoxidized, as shown in equation 3 where E represents the decrease of free energy which occurs in the reaction. This energy is used for the resynthesis of photolyt (equation 4). By summing equations 1 through 4 we get equation 5, the reduction of one molecule of flavin with one quantum of light.

A few words of explanation are in order here. A set of equations such as these are not intended to represent details of chemical mechanism. When we know the details, we will insert them. Of course, equation 3 must be chemically linked with equation 4. Of course, one can invent variations on this theme, in great variety, and add postulated detail, fitting various components of the respiratory chain onto various segments of these reactions. All we are trying to do here is to construct a skeleton-framework which we regard as correct in principle.

We expect difficulty in detecting reaction 5, because FH_2 and O_2 will back react. I have never been able to drive any O_2 into the argon gas phase by illuminating any amount of chloroplasts with any amount of flavin.

One detail which I have not inserted into equations 1 to 5 is the fact that the autoxidation of reduced flavin leads to the formation of H_2O_2. In such a back reaction one consumes twice as much O_2 as is formed. If the H_2O_2 is immediately decomposed by catalase, this might make no

difference. But if the catalase is inhibited so that H_2O_2 accumulates, we may see the flavin reaction more readily. If external oxygen is present, we see the overall reaction as a net O_2 consumption, equivalent to half the amount of H_2O_2 formed. If no external oxygen is present, however, we can't consume twice as much O_2 as is formed. Then we might expect an apparent dismutation: $FMN + 2\,H_2O \longrightarrow FMNH_2 + H_2O_2$. This is the kind of dismutation Warburg and Krippahl have described for TPN. In the case of TPN, one can accumulate much $TPNH_2$ and H_2O_2 because these substances don't back react readily at low concentrations. But $FMNH_2$ does back react with H_2O_2. However, the reaction of $FMNH_2$ with H_2O_2 is slower than the reaction of $FMNH_2$ with O_2. Though we can't stop the unnecessary part of the back reaction with a catalase inhibitor, we might be able to delay it.

The fluorescence band of oxidized flavin provides a very sensitive indicator of the oxidation-reduction state of flavin. With the fluorescence method one can follow the photoreduction and reoxidation of flavin, even with high ratios of chlorophyll to flavin. The details of the procedure have been described elsewhere[12].

The fluorescence-exciting light can be used as the photoreducing light to give a continuous record of the photoreduction of flavin on a recorder. Such a set-up gives tracings like those shown in Fig. 3. The level of

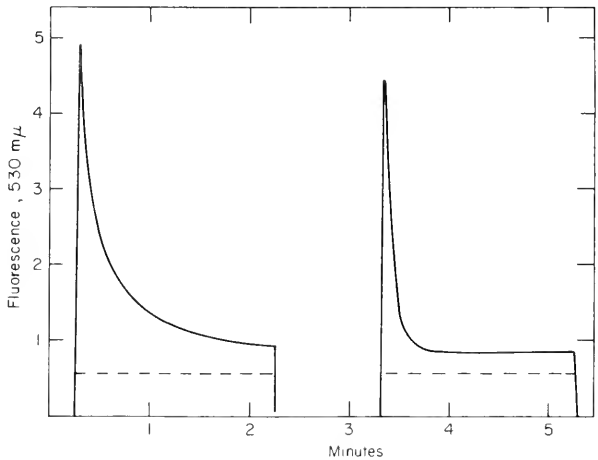

Fig. 3 Photoreduction of Flavin

scattered light is shown by the dashed line. The curves show how the fluorescence diminishes during illumination, but reappears after a dark interval.

We can also use visible light for the photoreduction, and follow the dark reoxidation by taking readings at intervals with a very small amount of fluorescence-exciting light. The measurements made in Fig. 4 were ob-

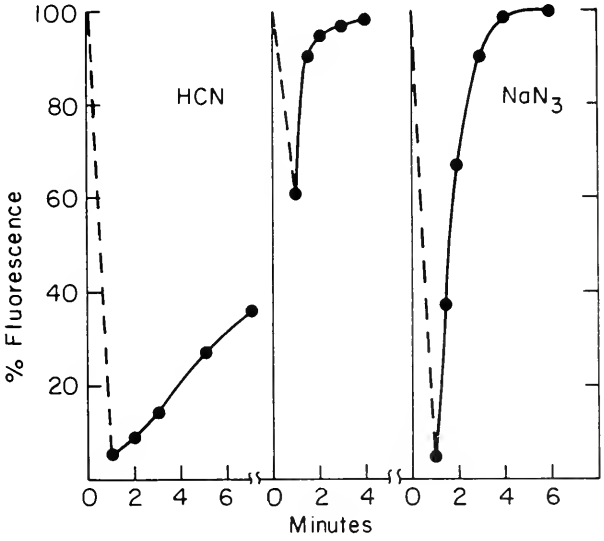

Fig. 4 Effect of Cyanide and Azide on Photoreduction and Dark Reoxidation of FMN

tained in this way. This experiment was done to show how reagents which inhibit catalase delay the unnecessary back reaction. Three different cuvettes were set up under argon with identical chloroplast plus flavin reaction mixtures, except that one contained cyanide in addition, one azide, and one, no inhibitor. All cuvettes were exposed to the same amount of light during the period indicated by the dashed line. Conditions used were selected to show how both cyanide and azide cause an apparent enhancement of the photoreduction of flavin. They assuredly do this by inhibiting the unnecessary back reaction. That is, the dismutation of flavin to reduced flavin and H_2O_2 appears to occur faster than the simple Hill reaction, because O_2 reoxidizes reduced flavin faster than does H_2O_2.

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To convince oneself that the reoxidation is inhibited by azide, one must compare rates starting from the same oxidation-reduction level of flavin. The much greater inhibition seen with cyanide than with azide is due to the fact that cyanide has a separate effect. With cyanide, the oxidant (O_2, H_2O_2 or oxidized FMN) eventually disappears under illumination, by oxidation of some reductant furnished by the chloroplasts. Such a consumption of oxidant occurs in the absence of cyanide too, but much more slowly. Azide does not enhance this disappearance of oxidant.

PHOTOPHOSPHORYLATION

All of the facts mentioned in the preceding section can be correlated with the effect of cyanide and azide on FMN-supported photophosphorylation, to prove that it is oxidized FMN which is required for the phosphorylation reaction. Thus cyanide causes a striking increase in the inhibitory effect of anaerobiosis on ATP synthesis, whereas azide has no such action.

If the photophosphorylation is carried out in CO_2-HCO_3^- buffer, the disappearance of acid during ATP synthesis leads to an uptake of CO_2 which can be followed manometrically. An experiment done in this way is shown in Fig. 5. In this experiment, two identical reaction mixtures were

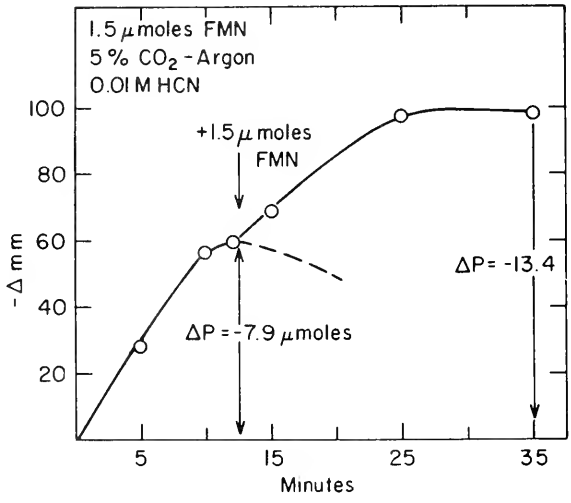

Fig. 5 Reactivation of Photophosphorylation

set up[12]. The contents of one of the vessels were analyzed for phosphate at the point where the manometric changes showed that ATP had reached a maximum (ATP-ase can also be followed with this procedure). At the same time, more FMN was added from the side-arm of the other vessel, to show how the phosphorylation was reactivated by this reagent. Examination of the flavin fluorescence at the first inhibition point revealed that the flavin was almost completely reduced, and that it remained reduced even when the light was turned off.

The important discovery of chloroplast photophosphorylation by Arnon appears to have lent great complexity to the Hill reaction. In connection with the complexity, I have a bad conscience, and I should like to make amends here. After Arnon had distinguished between stoichiometric and cyclic photophosphorylation, we suggested that one might differentiate a third kind, which we called oxidative photophosphorylation. Krogmann had made a study of indophenol supported photophosphorylation in my laboratory, and we were struck by the O_2 requirement for photophosphorylation supported by indophenol dyes. This was at a time when there was a great emphasis on anaerobic cycles with other cofactors for photophosphorylation, and I thought that the indophenol system must be different. I am convinced now that the apparent greater requirement of the indophenol system for O_2 under some conditions is due to the fact that this reagent is less readily oxidized than a reagent like flavin.

I would therefore like to withdraw the suggestion that we differentiate oxidative photophosphorylation from non-oxidative. I am not repudiating data, but the judgment about selecting a new name. One shouldn't complicate terminology beyond necessity. At the present time I think that all photophosphorylation is oxidative, in the sense that it occurs when a suitable reductant, formed in the light, is oxidized by the oxidized form of a Hill reagent. Seen in this light, photophosphorylation is a "substrate" phosphorylation[13, 14]. It appears to me that most of the phenomena studied in connection with photophosphorylation are compatible with this view, and that none disprove it.

I believe further that the catalytic cofactors function by a process which involves evolution and reconsumption of oxygen. Either of these two reactions may be rate-limiting for the cycle. It is consequently to be expected that an inhibitor of the oxygen-evolving step might be less effective under "anaerobic" than under "aerobic" conditions. This may not be the only

reason for diminished sensitivity to orthophenanthroline under anaerobic conditions, but it is surely one of them. The important fact to focus on is that sufficient concentrations of orthophenanthroline will always inhibit photophosphorylation substantially. In this connection, I regard it as unfortunate that the substituted ureas are so often used in preference to orthophenanthroline. These reagents have a limited solubility, and it may be impossible for that reason to add them in amounts sufficient to show the inhibition of the oxygen-evolving step.

I have tried to cover the field broadly, to elucidate the experimental basis for Warburg's theories about photosynthesis, and the manner in which these theories are applied to interpret the phenomena we study in isolated chloroplasts. We think of the chemical consequences of photon absorption in terms of chlorophyll and substrate first, rather than in terms of chlorophyll and the respiratory catalysts. The latter substances must function in the necessary back reaction or reoxidation. The cycling process in illuminated grana is visualized in terms of a release of O_2 from the activated CO_2-substrate, and a reoxidation of the reduced substrate by the O_2. This picture is consistent with the observed behavior in measured light of the gas exchange in intact photosynthesizing Chlorella, and is not refuted, so far as I can see, by any of the facts that have been accumulated with C^{14}, or with highly sensitive spectrophotometric measurements, or with any other analytical technique.

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UTILIZATION OF FAR-RED LIGHT BY GREEN ALGAE AND THE PROBLEM OF OXYGEN EVOLUTION

H. Gaffron, W. Wiessner and P. Homann

The overriding interest in recent research on photosynthesis of green plants (in contrast to that of bacteria) is the action of monochromatic light of different wavelengths and the nature of the Emerson effect. The latter is defined as the inefficiency of absorbed light beyond λ 700 mμ in promoting normal photosynthesis and the partial restoration of this low efficiency when light of shorter wavelengths is given simultaneously. The work summarized below gives a possible answer to the question whether the energy of far-red light is mainly lost unless combined with the energy contained in larger light quanta, or whether the absence of oxygen evolution only means that as the result of monochromatic illumination beyond λ 700 mμ another metabolic process takes over which is not connected with a visible gas exchange. There are no observations which prove that light of wavelengths longer than λ 700 mμ can by itself support a complete metabolic process in normal intact green algae. Such a metabolism ought to be sufficiently different from photosynthesis so as not to require a concomitant evolution of oxygen, i.e., it should resemble at least outwardly the photometabolism of purple bacteria. Photoreduction with hydrogen in adapted algae and photometabolism of acetate fulfill this postulate. We have found that both can be promoted very efficiently by far-red light alone.

PHOTOREDUCTION WITH HYDROGEN IN THE NEAR INFRARED

The gas exchange in a suspension of Scenedesmus cells in very weak bicarbonate buffer under H_2; 4% CO_2 is measured either manometrically or by means of a glass electrode which records pH changes. With unadapted cells a normal rate of photosynthesis can easily be demonstrated with visible light of a wavelength which is absorbed by the algae as weakly as light at λ 705 mμ. In the far-red of equal intensity oxygen evolution and carbon dioxide reduction proceed very poorly. When both lights are superimposed the yield is clearly much better than the sum of the yields obtained by each of the two lights alone.

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After adaptation in hydrogen the state of affairs is reversed, i.e., the efficiency in the shorter wavelength region is often much less than before and the far-red light has become surprisingly efficient. (Exact quantum yields have still to be determined.) The combined illumination does not produce the enhancement seen under aerobic conditions. Instead we often observed that mixing short and long wavelength lights seemed to lower the efficiency. Data published elsewhere by Bishop and Gaffron show that any conditions which aerobically diminish oxygen evolution, such as poisons, Mn deficiency, or genetic changes, leave the photoreductive CO_2 fixation as efficient in the infrared as it is in visible light. It follows that complete carbon dioxide assimilation into the usual reduced products is possible with near infrared light, provided that there is no need to eliminate the equivalent amount of oxygen as free gas.

ASSIMILATION OF ACETATE IN THE NEAR INFRARED

The alga Chlamydobotrys needs acetate and light for growth. Its respiration in the dark cannot replace the action of light. This organism, first described by Pringsheim and Wiessner and studied intensively by Wiessner, was subjected to the same tests as the Scenedesmus described above. But in contrast to the behavior of the adaptable algae there was no need for anaerobic treatment. Comparing the efficiency of acetate assimilation at various points in the shorter wavelength region (λ 680, 660, 620, 547 and 450 mμ) and at λ 705, 723 and 740 mμ, we found that in the region where in the better known green plants the efficiency of photosynthesis is high, that of acetate assimilation in this peculiar organism is low. It rises steeply, however, in the far-red when the chlorophyll a peak at λ 685 mμ has been passed.

Wiessner found earlier that in presence of carbon dioxide (which normally is not needed when the algae are grown in air) Chlamydobotrys can do some orthodox photosynthesis, just enough to keep it alive when it lacks acetate. We observed that the acetate assimilation in the infrared stops immediately when air is replaced by nitrogen. But half a per cent of carbon dioxide relieves the anaerobic inhibition. This relief occurs only when visible light of shorter wavelength is superimposed on the far-red light. Thus anaerobically we see a reversed Emerson effect. Under these conditions acetate assimilation remains poor with λ 723 mμ alone. Light absorbed in this region apparently does not produce the oxidative power needed to promote continuous

acetate assimilation. Light at λ 650 mμ alone showed the same inefficiency because the pigment which works mainly in the infrared (and presumably is needed for acetate assimilation) receives little energy. With both lights together the rate of acetate assimilation is drastically enhanced. (At high intensities of visible light we may assume a sufficient energy transfer so as to produce some deviations from the behavior just described.)

HYPOTHESIS

The simplest interpretation of the phenomena described above would be to say that the pigment absorbing at λ 700 - 740 mμ specializes in forming ultimately ATP through cyclic phosphorylation, and that it acts as pigment system I when coupled to activated pigment system II. (See the articles by Witt, Kok, Duysens in this book.) No theoretical picture suits the photometabolism of acetate in Chlamydobotrys better than that of two pigments, presumably two chlorophylls, which specialize in different photochemical reactions. The photosynthetic green algae rely mainly on that one which is indispensable for oxygen evolution. The acetate assimilating algae, however, depend under aerobic conditions mainly on an infrared absorbing chlorophyll which specializes in the formation of ATP.

But if the acetate assimilation has to proceed under anaerobic conditions the rate limiting factor for this metabolic process is its very low capacity for producing oxidative power at wavelengths beyond λ 700 mμ where as we assume ATP formation is the main consequence of the primary light reaction. Correspondingly the weak ATP formation at shorter wavelengths limits the rate of acetate assimilation even under aerobic conditions. It follows that illumination with light in the visible region, together with that beyond λ 700 mμ, is likely to promote the most efficient assimilation of acetate. That red light beyond λ 700 mμ supports ATP formation in chloroplasts better than oxygen production has already been shown by Hoch and quite recently by Arnon.

The strength of the coupling between systems I and II may vary from alga to alga according to whether they are in the habit of assimilating mainly carbon dioxide, or acetate, or able to switch reversibly between photosynthesis and photoreduction. A drop in overall efficiency can be expected whenever the specific metabolic processes connected to both systems are not properly adjusted to one another either in respect to reaction velocities or to the nature of the products.

H. Gaffron, W. Wiessner and P. Homann

OXYGEN EVOLUTION

Some time ago Bishop in our laboratory showed that the herbicidal action of DCMU and other substituted ureas is due to their ability to inhibit specifically the oxygen evolving process in green plants. Oxygen evolution is completely inhibited by DCMU concentrations as low as 10^{-6} M. The same concentration of this poison also stops the anaerobic acetate assimilation of Chlamydobotrys. This observation furnishes one important support for our assumption that an intact oxygen-evolving system is needed for the acetate photometabolism when no external oxygen is available.

Recent re-investigations on the light-induced evolution of hydrogen by adapted algae, first observed by Rubin and Gaffron, have led to the discovery that this hydrogen evolution in the presence of uncouplers of phosphorylation is nearly always accompanied by a simultaneous evolution of some oxygen. As Bishop reports elsewhere in this volume, the knallgas evolution by adapted algae (first seen in adapted Chlorella by Spruit) depends on an intact oxygen evolving system.

While the absorption of hydrogen during photoreduction is completely unaffected by DCMU, its evolution was found to be as sensitive to this poison as photosynthesis itself. Without going into details, we may say that a simultaneous production of hydrogen and oxygen, often in a ratio 2 : 1, under conditions when synthetic processes are severely curtailed, strongly suggest that a photolysis of water is the very first result of the cooperation between the primary processes in pigment systems I and II.

Where DCMU and the other substituted ureas interfere with the free flow of electrons within the presently accepted two pigment system is not known. There is unanimity that the inhibition disturbs the path of oxygen near the end point, where an oxidized photoproduct dismutates to free oxygen gas. Manganous ions apparently act as a part of the enzymatic system which releases oxygen, as Kessler has shown in our laboratory. Therefore Homann began to investigate the influence of the substituted ureas on an artificial photoreactive system where manganous ions play the role of very specific catalysts. In the reaction mixture under study, 2, 3 diketogulonic acid is photoxidized in the presence of light-excited flavin molecules, manganous ions and oxygen. The observed effect of the substituted ureas on this photoreaction was unexpected. Instead of reacting with

manganese in some manner as Gaffron had assumed, Homann discovered that it was the excited flavin molecules whose sensitizing actions were strangely altered. A short description of these experiments has recently appeared in "Science". Let us point out only the far-reaching implications which would arise if indeed these in vitro experiments had anything in common with the oxygen liberating reactions in green plants. No other sensitizing dyes showed remotely the affinity for the substituted ureas which flavins display in aerobic as well as in anaerobic light reactions. In several enzymatic dark reactions in which flavin is required for the activity of the enzyme, DCMU had no detectable effect. It is the light-excited flavin which falls prey to the specific affinity of the substituted ureas. This affinity results in an interaction with the electron transfer from the reducing substrate to the dye or in a protection against the quenching of the triplet state by oxygen or iodide, which may go together with an influence on the catalytic effectiveness of the manganous ions.

We would like to explain, of course, the parallelism we have found between the action of DCMU in our in vitro experiments and its well-established effects in vivo.

The problem we are up against is to find out how possibly an electronically activated flavin could be so intimately connected with that specific reaction sequence which leads to the release of free oxygen and which seems to be the exclusive prerogative of system II, if we accept the now prevailing ideas on the mechanism of photosynthesis.

ON THE INTERRELATION OF THE MECHANISMS FOR OXYGEN AND HYDROGEN EVOLUTION IN ADAPTED ALGAE

Norman I. Bishop and H. Gaffron

In 1942 Gaffron and Rubin discovered that anaerobically adapted cultures of the green alga, Scenedesmus obliquus, strain D_3, in the absence of carbon dioxide, produced molecular hydrogen in the light[1]. A few years later a similar process was found to be a part of the metabolism of certain photosynthetic bacteria[2]. In the intervening years only sporadic studies have been conducted on the process as carried out by green algae, but the process of hydrogen evolution in bacteria has been intensively studied by Kamen, Gest and a number of their students. As a consequence, it has been generally assumed that the mechanism of hydrogen production in the two different systems was probably identical. However, Spruit's studies on the anaerobic gas metabolism of illuminated Chlorella indicated that this was an assumption still open to question. He observed what appeared to be a simultaneous production of hydrogen and oxygen and hesitantly suggested that both gases arose via the photolysis of water[3]. However, he favored the alternate hypothesis that the two gases arose via different pathways, i.e., oxygen arose from an endogenous Hill reaction while hydrogen came from the dehydrogenation of some unknown hydrogen donor.

While studying the physiological characteristics of certain photosynthetic mutants recently induced and isolated in this laboratory, studies on the mechanism of hydrogen production in green algae have been resumed. It is the purpose of this communication to demonstrate the strong interdependence between the mechanisms for the production of hydrogen and of oxygen. If all the major synthetic reactions of Scenedesmus are eliminated or inhibited, the chlorophyll system will evolve upon illumination both molecular hydrogen and molecular oxygen. Any condition which is known to block preferentially the mechanism of oxygen evolution equally inhibits the mechanism for hydrogen formation.

Norman I. Bishop and H. Gaffron

GENETIC BLOCK OF OXYGEN AND HYDROGEN PRODUCTION

Recently, a mutant of Scenedesmus (mutant 11) has been described which, while unable to evolve oxygen either in photosynthesis or in the quinone-Hill reaction, is fully equipped to assimilate carbon dioxide via photoreduction[4]. Despite the presence of an active hydrogenase and the ability to utilize external hydrogen, adapted suspensions of this mutant do not evolve hydrogen photochemically. The capacity of the parent strain of Scenedesmus and mutant 11 to evolve hydrogen in the dark and in the light are compared in Fig. 1. The genetic alteration produces a two-fold effect: hydrogen is not evolved in the light and the small rate of hydrogen production in the dark (which is identical to the reaction of the parent strain) is abolished in the light.

Three other mutant strains resemble mutant #11. In the light they are neither able to evolve oxygen aerobically (these will be termed "oxygen mutants"), nor produce hydrogen anaerobically. Their capacities for hydrogen formation are summarized in Table 1.

Table 1. Gas Metabolism of Adapted "Oxygen Mutants" of Scenedesmus D_3

Conditions	Strains of Scenedesmus			
Observation Time	ScD_3 - Parent	ScD_3 - 47	ScD_3 - 40	ScD_3 - a'
	mm^3 Gas Liberated			
Dark 2 hours	4.2	3.1	5.2	5.2
Light 1 hour	20.4	0	2.4	0

1000 ul cells in 3 cc of 0.05 M phosphate buffer, pH = 6.5. Temperature = 25°C. Gas Phase = N_2. 0.2 cc 19% KOH in center well of each vessel. CCP (final concentration = 10^{-5}M) added after adaptation. Period of adaptation = 14 hours. Light Intensity = 900 lux.

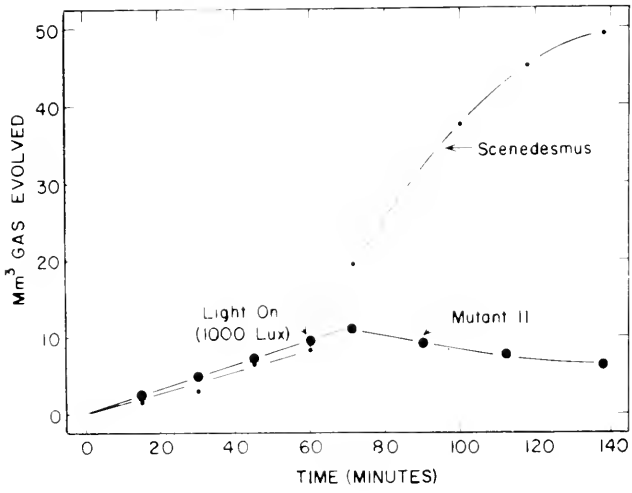

Fig. 1. Curves illustrating the dark and light production of hydrogen gas by H_2 adapted normal and mutant type Scenedesmus. O ——— O; "wild type" ● ——— ●, mutant II. See Table I for experimental conditions.

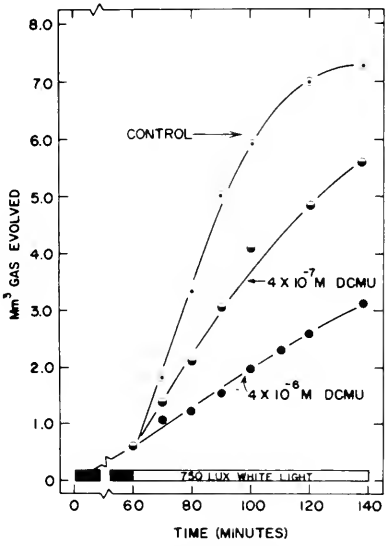

Fig. 2. The influence of DCMU on hydrogen production by adapted cells of Scenedesmus. O ——— O; Control, ● ——— ●, 4 x 10^{-7}M DCMU, ◐ ——— ◐, 4 x 10^{-6}M DCMU. Note break in the time axis during dark fermentation.

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Additional mutant strains of Scenedesmus have been isolated in which the genetic block resides in the carbon dioxide reducing side of photosynthesis (to be referred to as "carbon dioxide mutants"). All of these evolve oxygen in the quinone-Hill reaction and produce hydrogen in the light under the appropriate circumstances (Table II).

Table II. Gas Metabolism of Adapted Carbon Dioxide Mutants of Scenedesmus D_3

Conditions	Strains of Scenedesmus			
Observation Time	ScD_3 - 8	ScD_3 - 17	ScD_3 - 18	ScD_3 - 26
	mm^3 Gas Liberated			
Dark 2 hours	3.2	3.5	4.3	4.3
Light 1 hour	13.2	24.3	27.0	3.4

1000 ul cells in 3 cc of 0.05M phosphate buffer, pH = 6.5. Temperature = 25°C. Gas Phase = N_2. 0.2 cc 10% KOH in center well of each vessel. CCP (final concentration = 10^{-5}M) added after adaptation. Period of adaptation = 14 hours. Light Intensity = 900 lux.

From such observations the following question becomes apparent: How close a correlation exists between the capacity for oxygen evolution and that for hydrogen evolution in a normal cell? To explore this possible relationship further, other situations were examined wherein the mechanism of oxygen evolution is believed to be specifically blocked to determine if a parallel inhibition of photohydrogen production also occurs.

Norman I. Bishop and H. Gaffron

THE EFFECT OF INHIBITORS WHICH PREVENT OXYGEN EVOLUTION ON PHOTOHYDROGEN FORMATION

Poisons that specifically inhibit oxygen evolution while leaving photoreduction undisturbed have been known for some time[5]. The most effective ones in this respect are certain substituted phenylureas[6].

When such an "oxygen poison" is added to normal, adapted algae, little or no hydrogen is evolved in the light as compared to the control, regardless of the light intensity (Fig. 2). At a DCMU concentration of 6×10^{-5}M, where under aerobic conditions photosynthesis would be completely blocked, no hydrogen production occurs. At lower concentrations of DCMU (6×10^{-5}M and 6×10^{-7} M), photosynthesis and photohydrogen production are inhibited to the same degree.

Simazine, another specific inhibitor of oxygen production[7], also prevents hydrogen evolution. The results obtained with this herbicide were indistinguishable from those shown in Fig. 2 for DCMU.

MANGANESE DEFICIENCY AND HYDROGEN EVOLUTION

Manganese deficient cells of several algae are known to have a diminished rate of photosynthesis and of the quinone-Hill reaction while in algae that possess an adaptable hydrogenase the capacity for photoreduction of carbon dioxide with hydrogen remains unimpaired[8,9,10]. The ability of manganese deficient cells of Scenedesmus to produce hydrogen photochemically was tested and compared to normal cells (Fig. 3). Again the correlation is apparent that the capacity to evolve hydrogen in the light parallels that of the oxygen evolving system. As anticipated, the addition of 10^{-5}M $MnCl_2$ to deficient cells restores equally the capacity for hydrogen and oxygen evolution.

THE SIMULTANEOUS EVOLUTION OF HYDROGEN AND OXYGEN

Horwitz and Allen observed that the activation of a hydrogenase in an alga does not necessarily require a corresponding inactivation of its oxygen-evolving system[11]. Photosynthesis and photoreduction can proceed simultaneously as long as the emerging oxygen does not inactivate the hydrogenase. Thus the possibility had to be considered that what had been called an evolution of hydrogen was perhaps the evolution of both hydrogen and oxygen. If correct, the volume of gas produced during a light period should

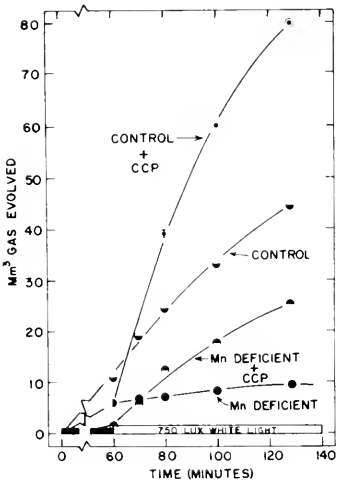

Fig. 3. The effect of manganese deficiency on the hydrogen metabolism of adapted Scenedesmus cells, with and without CCCP. O ——— O, Control + CCCP (10^{-5}M), ● ———●, control, ● ——— O, manganese deficient cells + CCCP, ● ——— ●, manganese deficient cells only. Note break in time axis during dark fermentation.

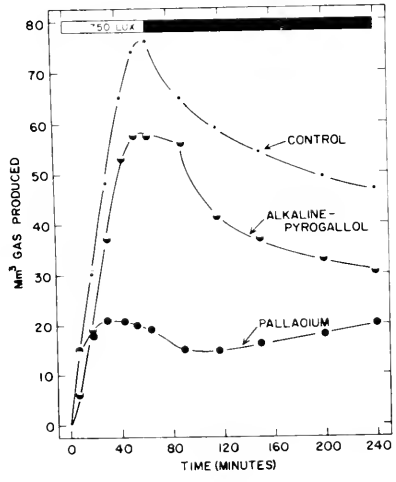

Fig. 4. Anaerobic gas metabolism of adapted Scenedesmus in the presence of alkaline pyrogallol and palladium. O ——— O, Control, ● ———●, alkaline pyrogallol in center well. ● ——— ●, palladium on scotch tape cylinder in center well.

not remain constant during a subsequent dark period, but rather diminish on account of the oxy-hydrogen reaction which ought to occur within adapted cells, i.e., an active hydrogenase would be present. Fig. 4 sums up the results of three experiments which agree fully with the assumption of a simultaneous appearance of the components of water. The curves indicate three ways by which oxygen disappears after it has been evolved. The shape of the upper curve indicates an oxyhydrogen reaction inside the cells themselves. This reaction starts very soon after the beginning of illumination and therefore forms a continuous part of the gas exchange in all three experiments during the entire period of observation. The middle curve represents readings obtained from a reaction vessel with alkaline-pyrogallol in the side arm. Here the greater part of the oxygen evolved should be removed separately, leaving hydrogen behind. The lower curve represents the same experiment but with a palladium catalyst in the center well[12]. Because of the immediate reformation of water there should be no measurable gas exchange at all. This is approximately true after the first ten minutes of illumination. There are two possible reactions which may be responsible for the appearance of the "extra" hydrogen observed in this experiment. First, excess hydrogen could be produced via a photochemical decomposition of an endogenous hydrogen donor, as it occurs in purple bacteria. Equally probable would be the oxidation of the internal hydrogen donor by oxygen or its immediate precursor. The end result, in either case, would be a shift in the hydrogen-oxygen stoichiometry in favor of the latter.

COMPARISON OF EMERSON ENHANCEMENT EFFECTS IN PHOTOSYNTHESIS, PHOTOREDUCTION, AND PHOTOHYDROGEN PRODUCTION

Following the initial experiments of Emerson and coworkers on the effect of the simultaneous addition of two selected wavelengths of light on the quantum yield of photosynthesis, it was widely believed that the Emerson "enhancement" phenomenon would be absent in photosynthetic systems in which oxygen is not produced, i.e., in bacterial photosynthesis and in photoreduction. Only recently have the experiments been performed wherein this "deduction" has been confirmed[13,14].

To explore further the hypothesis that the mechanism of hydrogen and oxygen evolution at least share steps in common, adapted suspensions of Scenedesmus were irradiated with two wavelengths of light, either singly or combined, which produced an enhancement effect in photosynthesis. Under such conditions an approximately equal enhancement of photohydrogen activity was observed. To the

contrary, but as expected from previous experiments, photoreduction showed no such stimulation and, at times, was even inhibited. These data are summarized in Table III.

TABLE III. COMPARISON OF THE EMERSON ENHANCEMENT EFFECT IN PHOTOSYNTHESIS, PHOTOREDUCTION, AND PHOTOHYDROGEN IN SCENEDESMUS D_3.

Wavelength of Exciting Light	Photoreduction (mm^3 gas uptake/hr)	Photosynthesis (mm^3 gas uptake/hr)	Photohydrogen Production ul H_2/hr
640 mu	54.6	32	26.6
705 mu	46.8	8	3.0
705 mu + 640 mu	80.6	47	38.0
Ratio $\frac{(640 + 705)}{(640)+(705)}$	$\frac{80.6}{101.4}=0.79$	$\frac{47}{40} = 1.18$	$\frac{38.0}{29.6} = 1.28$

INFLUENCE OF UNCOUPLERS OF PHOSPHORYLATION

In the original experiments of Gaffron and Rubin dinitrophenol (DNP) was observed to exert a twofold effect on the hydrogen metabolism of adapted cells: the dark formation of hydrogen was abolished but photohydrogen formation was markedly stimulated. This finding gave ample proof that two different systems were functioning in algal hydrogen metabolism. Because of the difficulty encountered with DNP in isolated chloroplast studies[15], m-chloro carbonyl cyanide phenylhydrazone (CCCP) was employed to study the role of phosphorylation in photohydrogen formation. Heytler recently discovered that this compound and other derivatives are potent uncouplers of phosphorylation reactions in general[16]. CCCP, like DNP, inhibits the dark formation of hydrogen and stimulates the light induced hydrogen production. A typical experiment demonstrating this is shown as part of Fig. 3. It is clear

that treatment with the uncoupling agent enhanced the rate 3-4 fold. Nevertheless, this enhanced rate of light induced hydrogen formation remains equally sensitive to inhibition by DCMU as the non-stimulated reaction. CCCP does not inhibit the quinone-Hill reaction or ferricyanide reduction by spinach chloroplasts.

Treatment of manganese deficient, and therefore poorly synthesizing, cells with CCCP stimulated the previously small rate of H_2 production (Fig. 3). And finally, addition of CCCP to adapted "carbon dioxide" mutants of Scenedesmus produced the increased rate of hydrogen evolution, but no effect at all was observed when adapted "oxygen" mutants were treated.

THE LACK OF INFLUENCE OF FLUOROACETATE AND IODOACETATE ON THE PHOTOHYDROGEN ACTIVITY OF SCENEDESMUS

Gest and coworkers have recently shown that hydrogen formation by Rhodospirillum rubrum occurs in part via an "anaerobic citric acid cycle" during which an exogenous hydrogen donor is oxidized to carbon dioxide and hydrogen[17,18]. One of their principle experimental findings to substantiate this hypothesis is that fluoroacetic acid inhibits hydrogen formation and citric acid accumulates. In preliminary experiments with monofluoroacetate and iodoacetate no inhibition of photohydrogen evolution was observed. For example, at concentrations of IoAA which abolished photosynthesis (3×10^{-4}M) no inhibition of photohydrogen activity occurred, but rather a stimulation. Similarly 10^{-3}M fluoroacetate, although not inhibitory to photosynthesis, stimulated but did not inhibit the production of hydrogen.

MASS SPECTROMETER ANALYSIS

Through the courtesy of Drs. B. Kok and G. Hoch some preliminary experiments were made on their mass spectrometer. The results confirmed our manometric observation (Figs. 1-4, Tables I and II) and in addition, showed that in normal, adapted Scenedesmus oxygen, as well as hydrogen, is produced. These data revealed a greater complexity and variability in the time course of rates and ratios for the appearance of the two gases. Similar observations were made a few years ago by Spruit[3].

CONCLUSIONS

The data reported here require an interpretation more complex than any previously offered for the mechanism of the production of hydrogen by adapted Scenedesmus cells. It appears quite evi-

dent that there is more than a coincidental relationship between a cell's capacity to evolve oxygen on the one hand and hydrogen on the other. The data obtained from mutants whose photosynthetic apparatus is known to be blocked in the oxygen pathway, from studies with known poisons of the oxygen system, and from manganese deficiency experiments support this conclusion. In addition, the simultaneous appearance of hydrogen and oxygen under adapted conditions, as determined manometrically and also with the aid of the mass spectrometer, adds further evidence to the hypothesis that both gases appear via a common mechanism. If indeed water is the substrate of both hydrogen and oxygen, then the ratio of the amounts of the gases formed should be 2. However, it is difficult, if not impossible, to determine this stoichiometry in a system which is oxygen starved. Spruit, in attempts to measure the hydrogen-oxygen ratio obtained the theoretical ratio of 2 only when he subjected adapted cells to freezing and thawing[3]. It was assumed that this procedure left intact and active only that portion of the photochemical apparatus responsible for the photolysis of water.

Assuredly the mechanism for the photochemical production of hydrogen by photosynthetic bacteria has been more thoroughly studied than the algal system, and from the work of Gest and coworkers good evidence is available as to the possible mechanism. Can it be assumed that the same or a similar mechanism is functional in the algal system? The following facts suggest that the answer to this question must be in the negative: (1) Hydrogen production in the bacterial system is suppressed by nitrogen and inhibited by ammonium salts, while in the algae the procees occurs equally well in nitrogen or helium; (2) Uncouplers of cyclic photophosphorylation inhibit the bacterial reaction but stimulate the process in Scenedesmus; (3) Hydrogen production is inhibited by all conditions known to interfere with oxygen evolution while the bacterial system is not inhibited by DCMU[19] and a manganese dependency would not be expected; (4) Fluoroacetate, which has been shown to be a potent inhibitor of the bacterial system does not hinder hydrogen evolution by Scenedesmus; (5) and finally, the hydrogen evolution in algae requires the two pigment system, i.e., shows the Emerson enhancement phenomenon, and so far no such response has been detected in the photosynthetic bacteria.

The theoretical meaning of these experiments on the simultaneous evolution of hydrogen and oxygen in the light becomes rather obvious when we consider that this reaction becomes stronger the more other chemical consequences of the primary process are suppressed. The best conditions are those where no reduction of

carbon dioxide occurs, no added compounds are present that could be used as hydrogen donors, and the phosphorylating system, which is assumed to function in a cyclic manner and thereby serve as a short-circuit between the photochemically produced reductant(s) and oxidant(s), is uncoupled by certain chemical agents.

Acknowledgement. This investigation was sponsored by the U. S. Atomic Energy Commission (AT-(40-1)-2687) and the Fels Fund.

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EFFECT OF LIGHT ON RESPIRATION

G. Krotkov

During this symposium it has been suggested by several speakers, that light has a depressing effect on respiration, as measured by oxygen absorption. In this connection some of the data obtained in our laboratory is of interest.

A detached tobacco leaf was placed in a closed system, and air, with the initial concentration of 100 ppm of CO_2 and 21% of O_2, was circulated over it. After a few minutes of illumination the lights were turned off. Figure 1 shows observed changes in the concentration of carbon dioxide around the leaf during illumination and subsequent dark period.

During illumination carbon dioxide content of air decreased until compensation point was reached. In darkness carbon dioxide content went up, its initial evolution being faster than later.

Figure 2 shows the rate of carbon dioxide evolution in darkness following previous illumination at different light intensities. Within the first minute of darkness there was a large outburst of carbon dioxide evolution, the magnitude of which was directly proportional to the previous light intensity. This was followed by a second outburst, which was much smaller and of longer duration. The steady rates of carbon dioxide evolution in the dark were reached after about six minutes.

When these experiments were repeated at concentrations of oxygen in the air varying between 2-47% the following changes in Figures 1 and 2 were observed:

(1) In light, the higher was the oxygen content of the air the higher was the compensation point. Figure 3 shows the relation between the two. Since at compensation point photosynthetic and respiratory rates are equal one can conclude that the rate of carbon dioxide evolution in light is increased with the increasing oxygen content of the air.

(2) In dark, the magnitude of the first outburst of photostimulated carbon dioxide evolution was also directly proportional to the oxygen content of the air. As is seen in Figure 4, at 2% oxygen, it was completely eliminated. On the other hand the final, steady rates of dark carbon dioxide evolution reached after six minutes were unaffected by oxygen concentration.

Comparable results have been obtained using several other kinds of leaves besides those of tobacco.

From the observation that the steady rates of carbon dioxide evolution in dark are not affected by oxygen, while those in light are, it has been concluded that evolution of carbon dioxide in light and in dark is the result of two different processes. While steady rates of carbon dioxide evolution in the dark represent true respiration, evolution of carbon dioxide in the light does not. Tentatively it can be called either a "photorespiration" or still better simply "carbon dioxide evolution in the light".

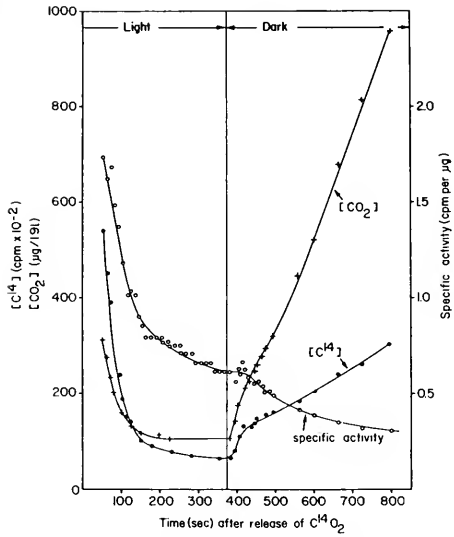

Fig. 1. Absorption and evolution of CO_2 and $C^{14}O_2$ by a detached tobacco leaf in light and dark.

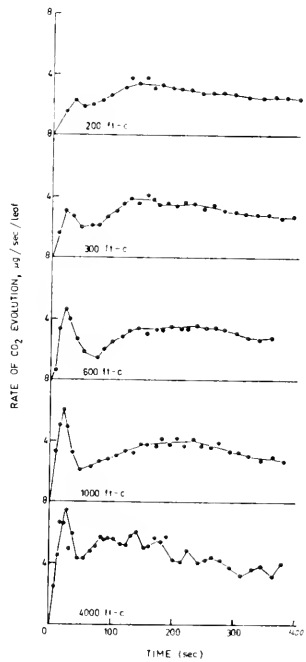

Fig. 2. Typical records of CO_2 production by tobacco leaf during the dark periods following illumination with different light intensities.

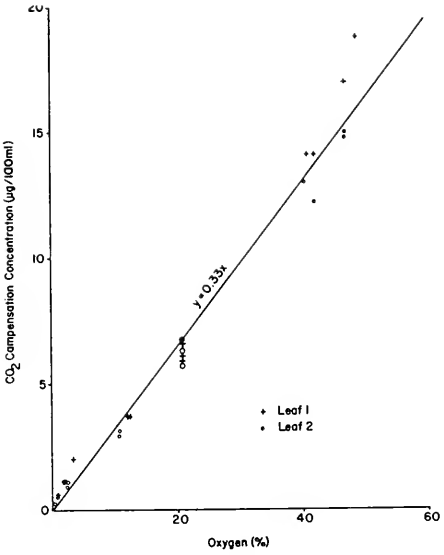

Fig. 3. Effect of O_2 concentration in air upon the compensation point in light.

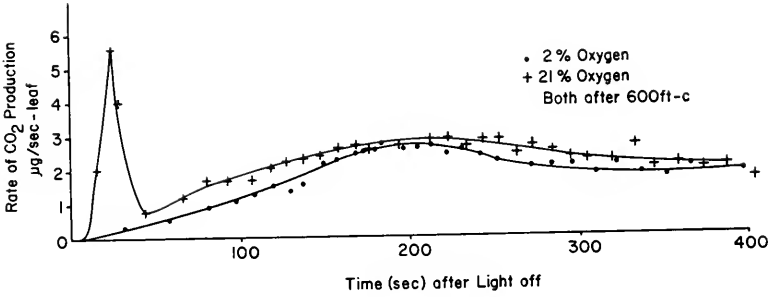

Fig. 4. Effect of O_2 concentration in air upon the evolution of carbon dioxide in dark.

VI. FUNCTION OF PIGMENTS AND PIGMENT COMPLEXES

AGGREGATED CHLOROPHYLL IN VIVO

S. S. Brody and M. Brody

In the introduction (Sections I and II) of this paper, we will cite some of the experimental findings which have been (or can be) interpreted as evidence for the existence of aggregated forms of chlorophyll in solution and in vivo. Although the in vitro systems which we examine involve primarily chlorophyll-chlorophyll interactions, while those in vivo most likely involve, additionally, chlorophyll-protein interaction, we contend that the homogeneous system is the principal one giving rise to the spectral properties seen in nature. Furthermore, we have attributed the numerous peaks in the red end of the absorption spectrum to a) the transitional distributions of chlorophyll aggregates during the formation of the pigment system, and b) to the distributions of chlorophyll aggregates characteristic of the various species of photosynthetic organisms in the "steady state".

In the body of this paper, (Sections III and IV) we will present some of the recent findings in our laboratories. Certain aspects of these lend further support to our contentions about the existence and role of chlorophyll aggregates in photosynthetic organisms.

I. EVIDENCE FOR THE EXISTENCE OF AGGREGATED FORMS OF CHLOROPHYLL IN SOLUTIONS AND IN LIVING SYSTEMS

A. The Aggregate in Solution

1. Evidence Based on Emission: Watson and Livingston (56), Lavorel (43), and Weber and Teale (59) have shown that the fluorescence yield of chlorophyll in solution decreases with increasing concentration above 10^{-3}M. By assuming the formation of non-fluorescent dimers at high concentrations, and calculating the amount of energy transferred to them, Weber (57) effectively accounted for the observed decrease in yield. The existence of the aggregated species predicted by these workers was experimentally demonstrated by Brody (4). He observed emission from an aggregated form of chlorophyll in solution, at 77°K, and reported the maximum of this band to be at 720 mμ. Its concentration and temperature dependence was described by Brody and Brody (9). Its low temperature emission was confirmed by Butler (16) and by Stensby and Rosenberg (52).

The latter workers noted (as had Brody (4)) that at room temperature the emission spectrum from concentrated solutions differed from dilute solutions, but they attributed the difference to deformation resulting from self-absorption of fluorescence, rather than to the presence of the aggregate. While the shift of the main emission band from 676 to 680 mμ - in going from dilute to concentrated solutions, respectively - can probably be attributed in large part to self-absorption, the shift in the "secondary peak" from 728 to 734 mμ cannot be. It cannot be, because at room temperature there is no significant absorption at wavelengths longer than 710 mμ; the shift has to be attributed to the formation of another fluorescent species.

It remains to be seen if the difference in position of the low temperature emission maximum in concentrated solutions - reported at 734 mμ by Stensby and Rosenberg (52), and at 720 mμ by Brody (4, 9) - arises simply from an instrumental artifact or from a more subtle source.

The lifetime of emission of the aggregate, at 77°K, has recently been redetermined by Butler (17); he reports it to be several nanoseconds, in which case, emission from the aggregate probably arises from a singlet state.

2. Evidence Based on Absorption Spectra: Trurnit and Colmano (53) have found that by compressing monolayers of chlorophyll it is possible to shift the postion of the red absorption maximum to 675 mμ - to correspond with the position of the maximum in Chlorella. The red shift is accompanied by increase in band width and change in ratio of red/blue absorption maxima - demonstrating that the in vivo absorption spectrum can be simulated solely by chlorophyll-chlorophyll systems.

In ethanolic solutions in which the chlorophyll concentration is greater than 10^{-2}M, broadening of absorption spectra can be observed at room temperature (6). A study restricted to the Soret band has indicated that similar broadening (24) occurs in chlorophyll dissolved in collodian when the concentration is increased from 10^{-6} to 5×10^{-4} M; these experiments could not be continued to higher concentrations because of high optical density.

Stensby and Rosenberg (52) have reported, however, that at room temperature they could not detect a change in the red absorption band of chlorophyll in ethanol - even though they worked with concentrations up to 8×10^{-3} M. They reported that at higher concentrations undissolved pigments were observed. We have recently repeated our measurements and agree with Stensby and Rosenberg that there is no appreciable change in the red region of the spectrum at concentrations of 8×10^{-3} M. However, at concentrations above 10^{-2} M, we find the same general features as we reported previously (6) (see Fig. 1 for our recently determined absorption spectrum.)

Stensby and Rosenberg do find evidence for the aggregate at low temperatures. Their absorption spectrum of an 8×10^{-3} M ethanolic solution of chlorophyll clearly shows an absorption band with a maximum at 705 mμ at

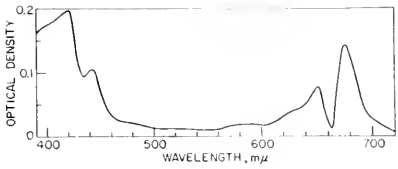

Fig. I. Difference in absorption between dilute (10^{-5}M) and concentrated (8×10^{-2}M) ethanolic solutions of chlorophyll a, at room temperature.

low temperatures, but they conclude that the chlorophyll dimer is formed upon cooling, although they point out that some of their data indicate the presence of dimer at room temperature also. (Another possibility is that upon cooling the dimer band undergoes a greater shift than the monomer band.)

3. Evidence Based on Fluorescence Excitation Spectra: From their studies of the action spectra for sensitization of chlorophyll fluorescence in highly concentrated solutions, Lavorel (43), and Weber and Teale (59) concluded that dimers of chlorophyll are present at room temperature. They attributed the "long wavelength decline" in efficiency of sensitization of fluorescence to an absorption band of a nonfluorescent chlorophyll dimer.

Low temperature (77°K) fluorescence excitation spectra have been determined with concentrated solutions of chlorophyll (7). That there is a marked difference between the spectrum for exciting emission at 720, and for exciting emission at 680 mμ, suggests that emission at 720 arises from a speices other than monomeric chlorophyll; in concentrated solution an aggregate becomes suspect. (To date, these excitation spectra have not been analysed to yield their various components.)

4. Evidence Based on Data from Other Sources: The formation of chlorophyll aggregates in acetone at concentrations in excess of 2×10^{-4} M was reported by Rodrigo (47). From measurements of light scattering, he determined the size of the aggregate as a function of concentration. At 9×10^{-4} M, the aggregate seemed to reach its average maximum size of three chlorophyll molecules.

Aronoff (3) has studied the colligative properties of highly concentrated solutions of chlorophyll in benezene and has shown that aggregation results in the formation of dimers, and that larger aggregates are of negligible concentration. It remains to be seen whether there is an unique size of aggregate formed in different solvents, e.g., dimers in benzene, trimers in acetone.

B. The Aggregate in Living Systems

1. Evidence Based on Emission: On the basis of the affects of heating and drying on fluorescence of green leaves, Seybold and Engle (49) proposed the existence of two forms of chlorophyll. They visualized most of the chlorophyll as being in a nonfluorescent, protein-bound state with a relatively small fraction dissolved in a lipid phase. Franck (25), founding his considerations on still other experimental work, presented a more detailed picture in which he suggested a high population of nonfluorescent, "protected chlorophyll" not directly connected with the photochemistry of photosynthesis, and a smaller population of fluorescent, "unprotected chlorophyll" in contact with water and capable of sensitizing photochemistry in vivo.

Support for the existence of more than one species of chlorophyll in vivo also comes from observations of fluorescence during the greening process in a wide variety of organisms, both at room temperature (27) and at 77°K (12, 31, 44, 48).

The discrepancy between the fluorescence yields, determined directly (41), and from measurements of fluorescence lifetimes (13), was also interpreted as evidence for the existence of fluorescent and nonfluorescent forms of chlorophyll in vivo (9). Brody and Brody (9) suggested, however, that the fluorescent form (emitting at 685 mμ) corresponds to a monomer, and that the "non-fluorescent" form (not contributing to emission at 685 mμ) corresponds to an aggregate.

The many similarities between low temperature emission spectra of photosynthetic organisms and concentrated solutions of chlorophyll have prompted the suggestion that chlorophyll aggregates are present in vivo (4, 9), e.g., Brody and Brody (9) suggested that the 720 mμ emission reported at room temperature by various workers (19, 30), corresponds to aggregated chlorophyll.

Lavorel (42) has shown that during the period of fluorescence induction in whole cells, there are changes in shape of the fluorescence spectrum. He has been able to resolve two fluorescence components - one constant and the other variable - the variable component has a maximum at 720 mμ (at room temperature); this corresponds exactly to the maximum of emission of the chlorophyll aggregate (4).

2. Evidence Based on Absorption Spectra: The origin of the concept of more than one form of chlorophyll in vivo is often associated with the attempt to interpret the absorption properties of purple bacteria. (19, 39, 55). The single absorption band with maximum at ~ 770 mμ - which is characteristic of bacteriochlorophyll in vitro - is the only form one obtains upon extraction of bacteriochlorophyll from organisms which exhibit one, two or three absorption bands in the living state. The bands, which differ in relative intensity, have maxima at about 800, 850 and 890 mμ. They have been

attributed to the presence of various sized pigments aggregates (39) or association of pigment with various proteins or lipoproteins (19,55). The relative intensity of the three peaks has been regarded as a manifestation of the concentration of the corresponding complexes.

Evidence for two forms of chlorophyll was actually given earlier for green plants than for bacteria by Albers and Knorr (1), who resolved two absorption maxima - at 683 mμ and 670 mμ. However, these workers considered the two peaks as representing different chemical states of chlorophyll rather than associated states of chlorophyll. Their early observations were confirmed by French and co-workers (27) and Brown and French (15) who used a derivative absorption spectrometer and resolved not only the absorption maxima at 673 and 683 but two other maxima at 694 mμ and 707 mμ. French and co-workers(28,29) have demonstrated these forms of chlorophyll in a wide variety of organisms.

Krasnovsky and Kosobutskaja (37) noted that when etiolated leaves are placed in bright light for one minute, the newly formed chlorophyll a exhibits an absorption maximum at 670 mμ. After several hours of illumination the maximum shifts to 687 mμ; Krasnovsky and Kosobutskaja attributed this shift to aggregation and binding of some of the chlorophyll. This interpretation has been further extended to bacteria and algae (40,54).

Induced changes in absorption have yielded spectra with maxima at 705, 678 and 660 mμ (18,36); these peaks have been attributed to an unknown pigment, chlorophyll a, and chlorophyll b, respectively. By low temperature absorption spectroscopy of leaves, Butler (16) has also shown the presence of the 705 mμ absorption band.

A summary of the absorption maxima cited above and the methods used to obtain them are given below.

Table I

Absorption Maxima of Chlorophyll Reported in Vivo, in mμ

Differential Absorption	707	695	683	673	
Induced Changes	705		678		
Greening Process		687			670
Absorption at Low Temps.	705				

3. Evidence Based on Excitation Spectra: Excitation of fluorescence of algae at low temperatures has shown that the absorption spectrum of the chlorophyll aggregate differs from the monomer (7, 16). Butler (16) found that one of the bands which gives rise to emission from the aggregate (at 720 mμ) has an absorption maximum at 705 mμ.

4. Evidence Based on Photosynthesis Measurements: The close correlation between the onset of photosynthetic efficiency and the shift of the absorption maximum of chlorophyll from 670 to 678 mμ - observed during the "greening" process in etiolated plants (37,50) - is suggestive of the need for aggregated chlorophyll in photosynthesis. While Smith, French, and Koski (50) proposed that the only photochemically active form is the chlorophyll with maximum at 678 mμ, Krasnovsky and Kosobutskaja (37,38) suggested just the opposite possibility. In addition, the latter workers proposed that the band with absorption maximum at 670 mμ represents the fluorescent, monomeric form while the other, non-active form with maximum at 678 mμ, is aggregated and nonfluorescent. They also chose to attribute the spectral shifts in the greening process to formation of aggregates rather than to the formation of chlorophyll-lipoprotein bonds.

It may be recalled that Weber and Teale (59) and Lavorel (43) had attributed the long wavelength decline in yield of fluorescence to the formation of nonfluorescent aggregates. Lavorel had, in addition, suggested that this explanation could also be applied to photosynthesis, i.e., that the long wavelength decline arose because of photosynthetic inactivity on the part of the nonfluorescent aggregate in vivo. Lavorel's suggestion was in full agreement with the observation of his contemporaries on photosynthetic action spectra. However, it has been subsequently shown that when excitation at short wavelengths is coupled with excitation at long wavelengths, full photosynthetic efficiency may be realized at the longer wavelengths (20,21,22) - "Enhancement Effect".

Based on their fluorescence emission observations - that the proportion of aggregate and monomer varies widely between different organisms - Brody and Brody (5,8,10) proposed that excitation of both monomer and aggregate are necessary for photosynthesis and attributed the presence of the long-wavelength decline in photosynthetic activity ("Emerson Effect") to lack of fulfillment of this requirement at the longer wavelengths. They also interpreted the enhancement effect in terms of cooperative aggregate and monomer action.

Systematic studies of the action spectrum of the enhancement effect were made by Govindjee and Rabinowitch (32), and Myers and French (46). They show the presence of two photochemically active forms of chlorophyll in vivo, with maxima at about 670 and 678 mμ.

Goedheer (31) has demonstrated that the development of photosynthetic capacity depends not upon concentration of chlorophyll but rather upon formation of the chlorophyll emitting at 720 mμ (as observed at low temperatures). This supports the hypothesis (5,8) that the aggregated form of chlorophyll is essential for photosynthesis.

II. MATERIALS AND METHODS

A schematic diagram of the photofluorometric apparatus we use is shown in Fig. 2. Uncorrected spectra are recorded graphically, and simultaneously

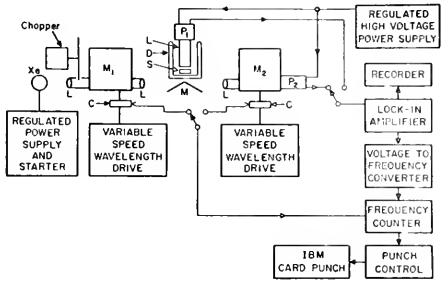

Fig. 2 Schematic diagram of spectrofluorometer: Xe = 1800 watt high pressure xenon arc lamp; L = lens; M_1 = monochromator for sensitizing fluorescence; M = mirrors; S = sample; D. = Dewar; L = light pipe; P_1 = Photomultiplier tube for measuring absorption; M_2 = monochromator for analyzing fluorescence; P_2 = photomultiplier tube for measuring fluorescence; C = commutator for triggering frequency counter and card punch.

corresponding values are punched onto IBM cards at 2 mμ intervals. The spectral response of the apparatus is calibrated by using a standard lamp, according to the method described by Stair, Johnston and Halboch (51). The intensity of the light incident on the sample is determined with a thermopile. These calibrations are incorporated into a computer program which (among other things) corrects all fluorescence and excitation spectra and plots the corrected curves. Thus, unless otherwise indicated, all spectra have been corrected on a quantum basis.

To deplete Euglena gracilis (Z) of its chlorophyll, the organism is cultured in total darkness for over 20 generations. In the present work, the term "age of the organism" will refer to the period of time of subsequent culturing in light.

III. RESULTS AND DISCUSSION

A. Photochemical Activity and Morphology of Cells which were Frozen and Thawed

Since many of the observations of fluorescence of algae are made with organisms subjected to extremely low temperatures, it was deemed desirable

to determine the effects of freezing on these cells. Studies were made of the morphological state of the photosynthetic apparatus, the photochemical activity of the chloroplasts, and the state of pigment aggregation.

1. Morphology: The structure of the chloroplast lamellae of Chlorella pyrenoidosa and Porphyridium cruentum was examined before and after freezing with the aid of an electron microscope. The algae, after being cooled to 77°K, were allowed to warm to room temperature, and were then immediately fixed with osmium tetroxide. The material was prepared with procedures similar to those given by Brody and Vatter (14).

The ultrastructure of the frozen Chlorella cells showed the following changes: extensive vacuolization of the cell cytoplasm, some damage to the nucleus (which probably accounts for subsequent failure of reproduction) and a small degree of vacuolization between the lamellae. Perhaps this last effect is related to the decreased activity of the frozen cells in carrying out Hill reaction.

2. Photochemical Activity: Hill reaction was measured with whole cells using quinone as the hydrogen acceptor. Manometric techniques were essentially similar to those described by Ehrmantraut and Rabinowitch (23). Two aliquots of cells were prepared; one was kept as a control, the other was cooled to 77°K, held at this temperature for about 10 minutes, and then thawed. Great care was taken to keep the cells in the dark. Hill reaction activity of the two aliquots was measured manometrically; each vessel contained 100 mm^3 of Chlorella pyrenoidosa, 2 mg of quinone, 0.05% KCL, and 1/20 M phosphate buffer (pH 6.5). (The quinone was freshly purified by steam distillation from 10N sulfuric acid, and later sublimated in vacuum.) The samples were flushed with nitrogen and brought to 20°C. Illumination was provided by a tungsten lamp.

It was found that cells which have been frozen and thawed still show Hill reaction but with a lowered efficiency. Figure 3 represents a portion of an experiment in which the production of oxygen by Chlorella cells has been determined. Essentially similar results were obtained by Horowitz (33), who used Chlorella, and 2, 6 dichlorophenolindophenol as Hill oxidant.

The major effects of freezing upon the Hill reaction are a lowering of yield by a factor of about 5, and an acceleration in loss of activity. But, since oxygen production as a function of light intensity and time of illumination is quite normal, it would seem that the photochemical apparatus is still relatively intact after freezing.

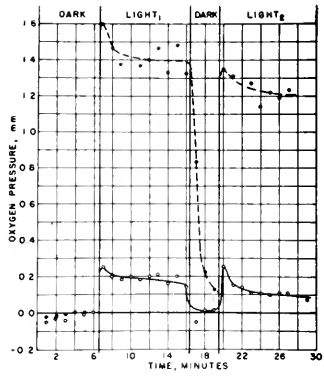

Fig. 3 Hill reaction of whole Chlorella cells. Control (closed circles) to be compared to cells which have been frozen and thawed (open circles).

3. State of Chlorophyll Aggregation: Comparison of "control" algal cells (Euglena, Chlorella) with those that had been frozen and thawed, revealed no appreciable change in absorption properties. Furthermore, the emission spectrum, the excitation spectrum, and the fluorescence yield remain characteristic for each temperature during the course of two to three cycles of freezing and thawing. However, after repeated cycles (five to six) a small shift in emission spectrum and diminution of intensity of fluorescence do occur. We feel that there is no evidence at the present time to indicate that a single rapid freezing of cells results in disruption of the normal distribution of monomeric and aggregated chlorophyll.

B. Emission from the Aggregated State in Vivo

(In this section, the wavelengths, given are not corrected for the spectral response of the apparatus.)

When young (2 day old) pale-green cells of Chlorella pyrenoidosa are excited with the 436 mμ Hg line, at room temperature, the resulting fluorescence spectrum consists of a main band with maximum at 685 mμ and a secondary band at about 720 mμ. (The latter represents a mirror image of the first vibrational band in the absorption spectrum of chlorophyll a.) (See Fig. 4) Excitation of young, pale-green cells of Euglena gracilis results in a similar emission spectrum.

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As the Chlorella cells age (to 1½ weeks) the fluorescence maximum of the main band shifts to about 687 - 690 mμ and the secondary band increases in intensity relative to the main band. (Figure 4.) Ageing in Euglena results in a similar effect, but with relatively greater increase in the secondary band in a much shorter period of time (see Fig. 5). By one week the main band

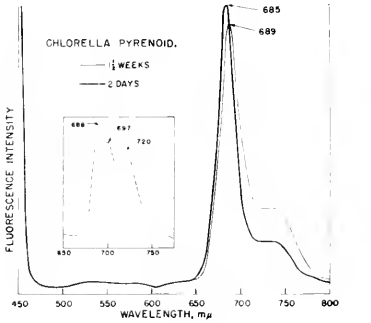


Fig. 4. Fluorescence spectra excited at 436 mμ. Room temperature spectra are given by solid curves. Spectrum at 77°K is given by dotted curve (for 2 day old cells).

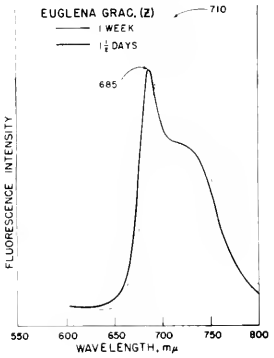


Fig. 5 Fluorescence spectra of 1 1/2 day and 1 week old cells excited at 436 mμ at room temperature.

has shifted and the secondary band has increased in intensity until only one wide band - with fluorescence maximum at about 710 mμ can be seen (Fig. 5). As the cultures age, the fluorescence of the main band - the monomer (685 mμ) increases in intensity, but the long wavelength - the aggregate - fluorescence increases at a more rapid rate.

The above experiments show that much more of the form contributing to fluorescence at 720 mμ is developed in Euglena than in Chlorella. Ochromonas danica and Monodus sp. were found to have properties similar to Chlorella, whereas Porphyridium cruentum was much more like Euglena .

That the shape of the emission spectrum in older Euglena does not arise primarily from re-absorption of fluorescence, is based on evidence of two types, firstly on relative fluorescence yield measurements, and secondly on the shape of the emission spectrum obtained upon excitation with various wavelengths of light. Relative yield measurements indicate that with ageing, the fluorescence of the monomer band (685 mμ) increases in intensity, but the fluorescence of the aggregate band (720 mμ) increases at a faster rate. As for the evidence based on the shape of the emission spectrum as a function of the wavelength of exciting light, the following was noted. Exciting the fluorescence of very young Euglena and Chlorella with various wavelengths of light does not seen to affect the shape of their emission spectra. Again, with older Chlorella, the shape of the emission spectra excited with

405, 436 and 545 mμ are relatively similar (although some small differences can be noted), but the fluorescence spectra obtained with older Euglena are markedly different - see Fig. 6 for 1½ day old cells and Fig. 7 for 1 week old cells. These data suggest that there are at least two different species

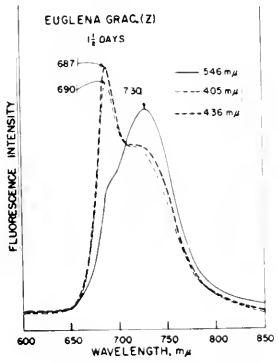


Fig. 6 Fluorescence spectra of 1 1/2 day old cells excited with wavelengths given above; excitation at room temperature.

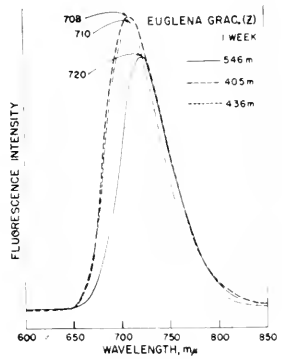


Fig. 7 Fluorescence spectra of 1 week old cells excited with wavelengths given above; excitation at room temperature.

of chlorophyll in Euglena which have different absorption spectra and which give rise to the 685 and 720 mμ emission bands.

Goedheer (31) in his study of greening bean leaves, has noted (with respect to the room temperature spectrum of a fully green leaf, and on the basis of induction phenomena) that the fluorescence around 720 mμ must be partially ascribed to emission by another form of chlorophyll rather than to scattering and re-absorption in the major peak.

The experiments described up to this point were made at room temperature, the following experiments involve also measurements at low temperature (77°K).

In Figs. 8, 9 and 10 may be seen a series of fluorescence spectra obtained with Ochromonas danica, age 2, 4 and 10 days, respectively. Except for the usual carotenoids, this alga contains no pigments other than chlorophyll a. The fluorescence in the blue-green region will be described in a separate section, below. First we turn our attention to the red region of the spectrum. In the curves obtained at 293°K, there is a small shift in fluorescence maximum with age, from about 690 mμ to 692 mμ, and an increase in fluorescence intensity (or relative fluorescence yield). The outstanding feature of the curves obtained at 77°K is the presence of two separate and distinct fluorescence bands in the red region. In the 10 day cells, the band

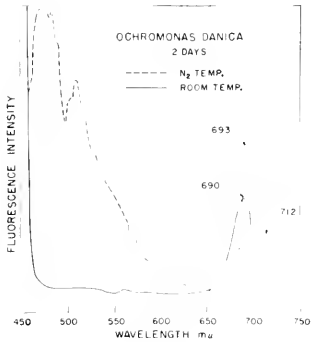


Fig. 8 Fluorescence spectra excited at 436 mμ.

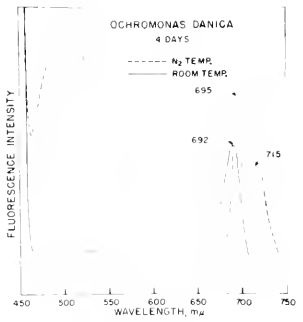


Fig. 9 Fluorescence spectra excited at 436 mμ.

at shorter wavelengths is displaced about 3 mμ, the one at longer wavelengths about 24 mμ from the maximum of the fluorescence band obtained at room temperature. (Freed and Sancier (26) have shown that a shift in absorption toward longer wavelengths should be expected upon cooling.) We will refer to the former as the monomer band, since it corresponds in position with the fluorescence band obtained with dilute solutions of chlorophyll; the latter will be designated the aggregate band, since it corresponds in position with the fluorescence obtained with concentrated solutions of chlorophyll. (4)

Measurements made at the temperature of liquid nitrogen also show (Figs. 7, 8 and 9) a shift in band maxima with ageing.

It can be clearly seen that in young Ochromonas the intensity of fluorescence from the monomer is greater than from the dimer; with age, both increase, but fluorescence from the aggregate increases at a faster rate. By 10 days the intensities of emission from the monomer and aggregate are about equal. This situation is also characteristic of higher plants, in which the intensity of emission from both bands is about equal in mature leaves during the entire growing season. (See Figs. 11 and 12).

The fluorescence properties of Chlorella are similar to those of Ochromonas and higher plants. However, as mentioned above, Ochromonas has only chlorophyll a; higher plants have in addition, chlorophyll b. Chlorella may be considered an extreme "shade" plant since it has a high proportion of chlorophyll b. In Fig. 4 it may be seen that in addition to the

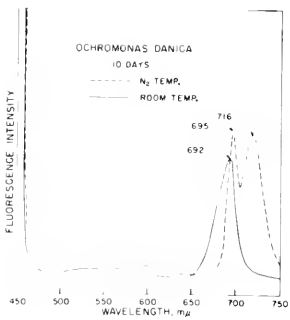


Fig. 10 Fluorescence spectra excited at 436 mμ.

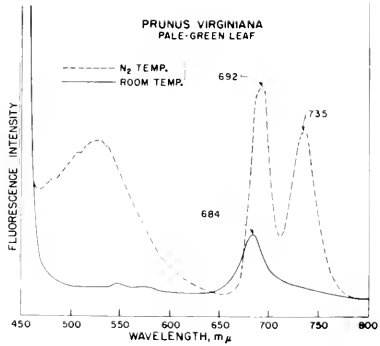


Fig. 11 Fluorescence spectra excited at 436 mμ.

monomer and aggregate bands characteristic of chlorophyll a, Chlorella exhibits a third band (or rather a shoulder) at 700 mμ. This may correspond to the aggregate band of chlorophyll b, or to the new band, described below in Section III - E. As the cells age, and the monomer and aggregate bands of chlorophyll a increase in size, this shoulder is obliterated.

It may be recalled that these "Chlorella - type" cells, when excited with various wavelengths of light at room temperature, yield essentially unchanged fluorescence spectra. Remembering also that the shapes of the emission spectra in Euglena do depend upon the wavelength of excitation, one might suspect that in "Euglena - type" organisms much more of the aggregate form is present. To investigate this possibility the following procedure was utilized. Etiolated cells were cultured in the dark on organic media for 3 days and then placed in light for specified periods of time.

This was done in order to obtain large quantities of pale-green cells. (Incidently, Euglena may be considered an extreme "sun" plant because of its low proportion of chlorophyll b to a.) In Figs. 13, 14 and 15 may be seen fluorescence spectra of 1, 2 and 3 day old Euglena respectively.

The total fluorescence from chlorophyll at room temperature is quite low in young cells, however, it can be seen that with ageing fluorescence intensity does increase. (In these experiments, emission from chlorophyll at room temperature was too small to measure in 24 hour cells.) Measurements made at room temperature also indicate a shift in monomer and aggregate maxima from 685 to 690 mμ, and 715 to 720 mμ, respectively. Although

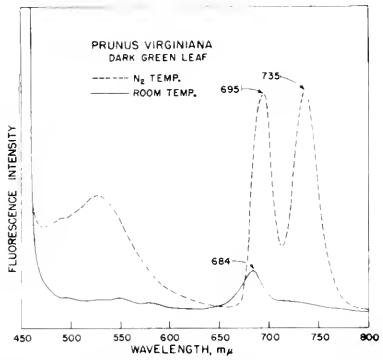


Fig. 12 Fluorescence spectra excited at 436 mμ.

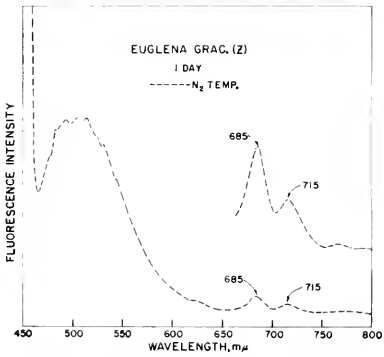


Fig. 13 Fluorescence spectra excited at 436 mμ.

emission from the monomer increases with age, the increment of aggregate emission far exceeds it. After about 48 hours in the light (the time, of course, depends upon such factors as the density of the culture, the intensity of light, etc.), the monomer band becomes just an inflection on the short

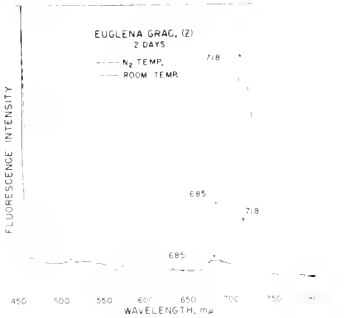


Fig. 14 Fluorescence spectra excited at 436 mμ.

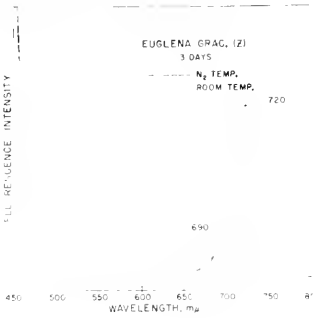


Fig. 15 Fluorescence spectra excited at 436 mμ.

wavelength side of the aggregate band and is soon completely masked. Consequently in Euglena, aggregate fluorescence exceeds monomer fluorescence during the major portion of the organism's life. These fluorescence characteristics also obtain for Porphyridium. It is of interest to note, that in the one organism of this type that we have examined - Porphyridium - the "Emerson Effect" occurs at relatively short wavelengths (20,21,22); Brody and Brody (5,8,10), suggested that the disproportion of aggregate and monomer at longer wavelengths does not permit the two photochemical reactions which they postulated, to occur efficiently.

Now let us return to our observation of emission in the blue-green region of the spectrum, which is especially evident at low temperature (see Figs. 8 - 15). Although this emission is apparent in all the organisms we have examined, only in Euglena is there a clear cut pattern in its appearance. (We did not study this phenonenon in detail in the other organisms, and in Ochromonas we have not been able to get consistent data.) In young Euglena, in which there is little chlorophyll, emission in the blue-green region is strong. As the cells age and more chlorophyll is formed, there is a corresponding decrease in blue-green emission (which may be only relative - although preliminary data suggest there is actually a dimunition in yield). This fluorescence in Euglena was attributed to carotenoids by Brody and Linschitz (12) on the basis of similar emission and action spectra for carotenoids extracted from Euglena with alcohol. It should be noted that Goedheer (31) has attributed fluorescence in this region in greening bean leaves to FMN and DPNH derivatives.

C. Size of Chlorophyll Aggregates in Vivo

The marked similarity between the spectral properties of chlorophyll in vivo and aggregates in vitro has already been noted (4, 9). In this section we assume that, except for the species giving rise to emission at 687 mμ, all of the chlorophyll in vivo is present in the form of molecular aggregates. On this basis, an effective size for the aggregate is estimated from the position of the aggregate emission maximum at 77°K. It is further shown how the size of the aggregate varies with the age of the organism.

After dark grown Euglena has been in light for several hours, there is noted, in addition to fluorescence from the chlorophyll monomer (maximum 687 mμ), a second emission maximum at 717 mμ (77°K). After Euglena has been in light for still longer periods of time, the position of the long wavelength emission maximum shifts further toward the red end of the spectrum, until - after 80 hours - it reaches 732 mμ. The position of the long wavelength emission maximum as a function of time in light is shown in Fig. 16, where a smooth curve has been drawn through the experimental points.

To estimate the size of the aggregate, an expression derived by McRae and Kasha (45) is used:

$$\tilde{\nu}''_{A_1} - \tilde{\nu}''_{A_2} = 4(N-1)m^2(1+\cos^2\alpha)/NhcR^3 \qquad \text{Eq. (1)}$$

N = number of chlorophyll molecules in the aggregate; $\tilde{\nu}''_{A_1}$ and $\tilde{\nu}''_{A_2}$ = absorption maxima of aggregate; α = angle between the axis of the aggregate and the planes of the molecules forming the aggregate; R = distance between charge centers; c = speed of light; h = Planck's constant; m = transition moment.

In the present analysis, it is assumed that the changes in spectroscopic

properties which accompany greening, result solely from changes in the size of the aggregate, and that the geometry of the aggregate (R and α) remains constant.

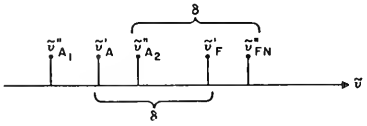
Equation 1 will be re-written in terms of emission, since the data which are available for the aggregate in vivo are for emission rather than absorption. To accomplish this we take the wave number difference, δ, between the absorption and emission maxima of the monomer and set it equal to the wave number difference between the absorption and emission maxima of the aggregate, i.e.

$$\tilde{\nu}'_A - \tilde{\nu}'_F = \delta = \tilde{\nu}''_{A_2} - \tilde{\nu}''_{F,N}$$

In this expression subscripts A and F denote absorption and fluorescence, respectively, and primes and double primes denote monomer and aggregate, respectively. Assuming there is symmetrical splitting of the monomer absorption band upon aggregation, it can be shown that Eq. 1 may be re-written in terms of emission maxima, in the following way:

$$\tilde{\nu}''_{F,N} = \tilde{\nu}'_F - 2\,(N-1)m^2\,(1+\cos^2\alpha)/NhcR^3 \qquad \text{Eq. (2)}$$

A pictorial representation of our notation is shown below.



The long wavelength emission maximum-which is observed at low temperatures with Euglena exposed to light for only a few hours,- is assumed to arise from the smallest possible aggregate - namely, a dimer (N=2). Using this value for $\tilde{\nu}_{F,N}$, the term $m^2\,(1+\cos^2\alpha)/hcR^3$ in Eq. 2 can be eliminated to yield our working equation:

$$(\tilde{\nu}'_F - \tilde{\nu}''_{F,N}) = 2(\tilde{\nu}'_F - \tilde{\nu}''_{F,2})(1 - 1/N) = A(1-1/N) \qquad \text{Eq. (3)}$$

in which $\tilde{\nu}'_F$ and $\tilde{\nu}''_{F,2}$ are constants.

Since we are dealing with a distribution of molecular aggregates, it should be recognized that the emission maximum ($\tilde{\nu}''_{F,N}$) determined experimentally is an average value, which can be represented by the following expression:

$$\overline{\tilde{\nu}''_{F,N}} = \sum_{N=2}^{\infty} \tilde{\nu}''_{F,N}\,[N] \Big/ \sum_{N=2}^{\infty} [N] = \tilde{\nu}'_F - A\{1 - \overline{[1/N]}\},$$

where $[N]$ is the concentration of aggregates of Size N, and A is as defined in Eq. 3. An effective size for the aggregate $1/\overline{[1/N]}$ (slightly greater than the true average size N) can be calculated with the aid of Eq. 3. For convenience, let $1/\overline{[1/N]} = \eta$. To determine A the following data obtained

from Fig. 16 is used: $\tilde{\nu}''_{F,2} = 13,947\ cm^{-1}$ ($\lambda''_{F,2} = 717\ m\mu$) and $\tilde{\nu}'_F = 14,566\ cm^{-1}$ ($\lambda'_F = 687\ m\mu$), so that $A = 2(\tilde{\nu}'_F - \tilde{\nu}''_{F,2}) = 1218$. The effective value of $\mathcal{N}$ (calculated from Eq. 3) as a function of $\tilde{\nu}''_{F,N}$ is shown in Fig. 17. The longest wavelength for the emission maximum in our experiments is $\lambda''_{F,N} = 732\ m\mu$ ($\tilde{\nu}''_{F,N} = 13,661\ cm^{-1}$) which yields $\mathcal{N} = 3.77$.

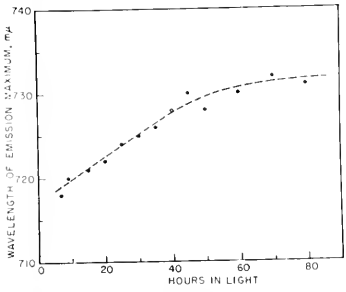


Fig. 16 Data for Euglena at 77°K; long wavelength emission maximum as a function of age of cells - excited at 436 mμ.

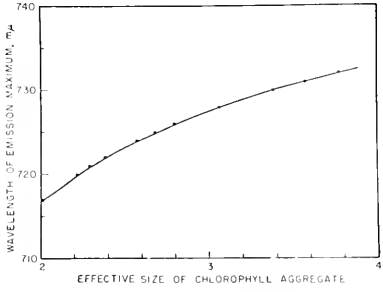


Fig. 17 The position of the long wavelength emission maximum (at 77°K) as a function of the size of the aggregate (based on data obtained from Euglena).

The long wavelength absorption maxima of the various aggregates can be calculated from their emission maxima, using the previously defined expression $\delta = \tilde{\nu}_A - \tilde{\nu}'_F = 14,793 - 14,566 = 237\ cm^{-1}$. Representative absorption maxima calculated in this way are listed in Table II.

Table II

Emission Maximum			Absorption Maximum	
$\tilde{\nu}$ (cm^{-1})	λ (mμ)	$\mathcal{N}$	$\tilde{\nu}$ (cm^{-1})	λ (mμ)
14,556	687	1	14,793	676
13,947	719	2	14,184	705
13,661	732	3.77	13,898	719
13,338	750	∞	13,575	736

The long wavelength shift of emission maximum which occurs during greening in Euglena, is accompanied by a broadening of the red absorption band as well as a long wavelength shift of the absorption maximum.

The spectral transformations in Euglena can be interpreted in several ways: 1) there is an association of chlorophyll with different proteins 2) there is an aggregation of chlorophyll molecules 3) both phenomena may occur simultaneously. Whereas chlorophyll molecules are probably bound to the protein portion of the lamellae, we believe that it is primarily the inter-

action between chlorophyll molecules, rather than between chlorophyll and protein molecules, which gives rise to the spectral transformation observed in vivo. While the heterogeneous interaction is possible, the fact that many spectral properties found in vivo can be simulated by protein-less solutions of chlorophyll lends support to our contentions.

In the present analysis it was assumed that first long wavelength emission band to appear (at 717 mμ) arises from the smallest possible aggregate. This assumption is supported by the following: if the distance between molecular centers were the main parameter determining the position of the emission maximum, then there would be a continuous shift in maximum starting from the position of the monomer peak. However, a discontinuous shift of 32 mμ is observed between the position of the monomer maximum and the shortest wavelength at which emission from the aggregate occurs On the other hand, the discontinuous shift (which actually obtains) is to be expected if a small aggregate is formed. As the concentration of larger aggregates increases, a continuous shift in the emission band is to be anticipated because the displacements between maxima decrease rapidly with increasing size, e.g., the displacement between dimer and trimer maxima is only 9 mμ.

We are led to believe, therefore, that the spectral transformations observed in Euglena (and other organisms) represent changes in distribution of monomer, dimer, trimer, etc., and that this distribution undergoes a considerable modification between the time of initial formation of chlorophyll and attainment of a "steady state". The steady state distribution seems to depend upon the genus and species of the organism ( ). If these changes in distribution do occur, they would explain in large part the diversity of the reported maxima for the "various forms" of chlorophyll in vivo (See Table I).

By letting N go to infinity in Eq. 3, we calculate for $\tilde{\nu}''_{F,\infty}$, a limiting value of 13,338 cm^{-1} ($\lambda''_{F,\infty}$ = 750 mμ). The corresponding absorption maximum for an infinitely large aggregate, calculated from $\tilde{\nu}''_{F,\infty}$, (see Table II), turns out to be 736 mμ, a value which compares favorably with the reported absorption maximum of large microcrystals of ethyl chlorophyllide a - 740 mμ (34). This agreement lends support to our assumption about R, α and the value taken for $(1+\cos^2\alpha)/R^3$, and also indicates that the intermolecular dimensions in the crystal and the aggregate are similar.

It is of interest to note that the absorption maximum we calculated for dimeric chlorophyll in vivo (705 mμ), corresponds to the wavelength reported for the following kinds of studies: changes in absorption of irradiated organisms, (18, 36, 60), differential absorption (15, 28, 29), and low temperature absorption spectroscopy (16).

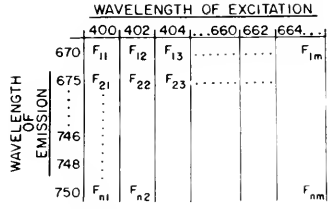
The shift in emission maximum from 719 mμ to 732 mμ (measured at 77°K) which occurs during the greening process in Euglena, can result from the

formation of aggregates with a final effective size 3.8 molecules (see Table II). This size is somewhat larger than the three molecules given by Rodgrigo (47) who, however, made his measurements with solutions of chlorophyll at concentrations lower than those found in vivo. Some of the possible molecular weight distributions, as well as mechanisms of aggregation - which give rise to the effective sizes we have found, will be discussed elsewhere.

D. Number of Florescing Forms of Chlorophyll in Euglena

The broad emission band observed in Euglena most likely corresponds to an envelope of several overlapping spectra. Before an evaluation can be made of the contribution of each fluorescing component to the envelope it is advisable to determine the number of components giving rise to the fluorescence envelope. Weber (58) has described a method for enumerating the components in such complex systems on the basis of fluorescence spectroscopy.

Weber's method essentially consists of setting up a matrix as shown below, where F_{nm} is the intensity of fluorescence obtained at wavelength n, upon



excitation at wavelength m. All possible 2 x 2, 3 x 3, 4 x 4, etc., determinants are evaluated. When all the 2 x 2's are zero, there is only one fluorescent form present; when the 3 x 3's are zero, there are only two fluorescent forms, and so on. In our experiments the measurements of $F_{n,m}$ each have an estimated error of 5%, therefore, when we say the determinant is zero, it means it is zero within our experimental error. The fluorescence spectrum under study covers the spectral range 670 to 770 mμ. The intensity of emission is measured at 5 mμ intervals over this range so that as many as 21 excitation spectra can be obtained. The excitation spectrum which corresponds to a particular wavelength in the emission spectrum is punched, at 2 mμ intervals, onto IBM cards, resulting in 125 to 175 points for each excitation spectrum.

The matrixes were calculated on an IBM 7090 computer. There is no problem in doing all the possible 2 x 2 matrices. However, because of the long time it takes to evaluate all the possible 3 x 3 and 4 x 4 matrices in a 21 x 150 matrix, some of the data had to be eliminated.

Fluorescence excitation spectra were determined, at room and liquid nitrogen temperatures, for dark grown Euglena gracilis which had been allowed to green for periods of time ranging from 6 to 80 hours. With these

cells, none of the 2 x 2 matrices vanished completely at either temperature. For some of the experiments made at room temperature, all the possible 3 x 3 matrices vanished - but for other experiments at room temperature there were 3 x 3 matrices which did not vanish. It remains to be seen under which conditions only two emission bands are possible at room temperature. Since, with the experiments made at 77°K, none of the 3 x 3 matrices vanished completely, there exist more than 2 fluorescent components at this temperature. Although our preliminary experiments indicate that all the possible 4 x 4 matrices vanish completely at both temperatures (implying the existance of no more than three emission bands) we wish to re-confirm these data.

One of the three fluorescing components corresponds to the chlorophyll monomer. If one of the other bands corresponds to a dimer, then the third remaining band would have to be a tetramer - since our calculations (in section III -C) indicate that the chlorophyll aggregate increases until it attains an effective size of 3.8 chlorophyll molecules. We must assume, therefore, that either a trimer exists - but is nonfluorescent, or that it does not exist - the formation of larger aggregates involving condensation of dimers to form tetramers.

E. Low Temperature Emission Spectroscopy of Dilute Chlorophyll Solutions

Low-temperature emission spectroscopy of dilute solutions of specially prepared chlorophyll a reveals the presence of three bands(11, 61). In Fig. 18 curve II, may be seen the emission spectrum of this chlorophyll, 10^{-5}M in acetone, excited with blue light (436 mμ); in addition to the anticipated maxima at 671 and 725 mμ, there is another band with maximum at 698 mμ. At room temperature this new band is not observed, and the emission spectrum is similar to others previously reported for chlorophyll a (19).

We have found that crystalline chlorophyll a, prepared according to either the method of Jacobs, Vatter and Holt (35), or Anderson and Calvin (2) yields, upon excitation only two emission bands at 77°K. (See curve I in Fig. 18). The chlorophyll with which we have observed the additional band is obtained by washing the crystalline chlorophyll-prepared by either of the above methods - exhaustively with petroleum ether. Preliminary measurements of the emission lifetime of the new band show it to be shorter than 10^{-6} sec.

There are no appreciable differences that one can observe at room temperature in the absorption spectra or extinction coefficients of the chlorophyll preparations which do and do not show emission at 698 mμ. This third band with maximum at about 698 mμ has been observed in the following solvents at 77°K: acetone, benzene, ethanol, ethyl ether, and pyridine. At this point it might be well to recall the third emission band seen in young Chlorella, at 77°K (See Fig. 4), its maximum at 697 mμ (uncorrected) is close to the

position of the new band reported here for dilute solutions of chlorophyll.

We have made a preliminary survey of the materials which quench the emission at 698 mμ. These include nitrobenzene, p-phenylenediamine, and the 'residue' of the petroleum ether washings of the crystalline chlorophyll. The petroleum ether 'residue' completely quenches the emission at 698 mμ; adding 'residue' to the same sample which gives curve I (Fig. 18) yields curve III (Fig. 18). Materials which do not quench the emission are commerical β carotene and vitamin K, as well as crude xanthophylls and carotenes extracted from spinach.

A comparison of curves II and III shows that the petroleum ether 'residue' modifies the main emission band by decreasing its width, shifting its emission maximum to shorter wavelengths and increasing its intensity. By "subtracting" curve III from curve II (using as a condition no negative values), we have obtained the emission spectrum of the "698 mμ species" Fig. 19. In addition to the maximum at 698 mμ, there is an even larger maximum at 680 mμ. We presume that this difference spectrum corresponds to one band with two maxima, but it is possible that the two peaks represent two bands.

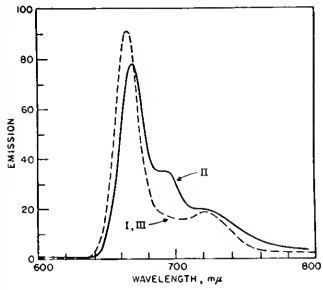


Fig. 18 Emission spectra for specially prepared chlorophyll in acetone (see text) at 10^{-5}M. Curve I - emission obtained with chlorophyll before purification. Curve II - spectrum showing new emission band, Curve III - same sample as for curve II but with quencher added. All excitations at 436 mμ.

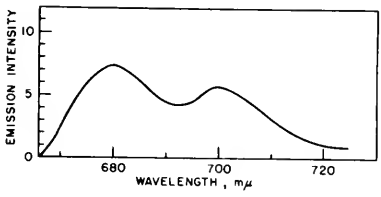


Fig. 19 Spectrum obtained by taking the difference between curves II and III in Fig. 18.

We are in the process of identifying the petroleum either 'residue', and of determining if there is more than one naturally occuring quencher in chloroplasts. So far, we have found that the 'residue' of the petroleum ether washings has some of the properties of a terpenoid quinone (such as plastoquinone or coenzyne Q).

Since the naturally occuring quencher ('residue') is not readily removed from chlorophyll a, even after repeated chromatography, it may be that it is strongly complexed with chlorophyll. This contention is supported by the fact that even in very minute amounts it will quench emission at 698 mμ. The ease with which this excited state is quenched is significant for it suggests that the excited state may be effective in sensitizing photoreactions.

F. Excitation Spectra in Solution and In Vivo

In Fig. 20 may be seen an excitation spectrum (for emission at 730 mμ) determined with Euglena (in light 80 hours) at 77°K; it contains a band with maximum at 708 mμ. The general features of this spectrum are similar to the excitation spectrum (for emission at 736 mμ) determined with a concentrated solution of chlorophyll in pyridine (10^{-2}M) at 77°K; in Fig. 20 may be seen a band with maximum at 718 mμ which we feel corresponds to the 708 mμ band in vivo. Similar experiments with dilute solutions of chlorophyll yield excitation spectra (for emission at 726 mμ) which do not contain the band at 718 mμ, (or for that matter, any bands at all between the monomer absorption maximum at 680 mμ and the emission maximum at 736 mμ); see Fig. 20.

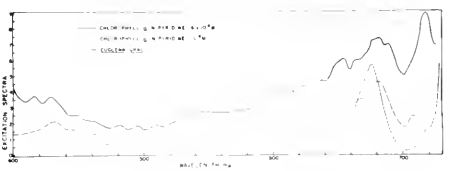


Fig. 20 Excitation spectra for dilute and concentrated solutions of chlorophyll a in pyridine, and Euglena, at 77°K; wavelengths being monitored given in text.

With very young Euglena (in light only a few hours) the band at 708 mμ is only a small shoulder. All of our data on the 708 band in vivo has not yet been analyzed in a quantitative fashion, however, it appears at least qualitatively - that the magnitude of this absorption band and the intensity of the emission band between 720 and 730 mμ increase in a parallel fashion. In light of the above findings, we would like to suggest that it may not be necessary to assume a colorophyll-cytochrome complex (16) to account for

the absorption in vivo at 705 mμ; chlorophyll aggregates in solution (in the absence of cytochrome) exhibit this band.

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PROPERTIES OF CHLOROPHYLL PROTEIN ISOLATED FROM LEAVES OF CHENOPODIUM ALBUM

Atusi Takamiya, Hirosi Obata and Eijiro Yakushiji

INTRODUCTION

Various attempts have been made to disintegrate chloroplasts into its constituent units and obtain chlorophyll in the nearest-to-nature state as it occurs in the photosynthetic apparatus. The isolation of chloroplastin[1, 2], protochlorophyll holochrome[3] and quantasome[4] may be regarded as the most prominent achievements along this line of approach. The purpose of the present report is to make another addition to this group of chlorophyll proteins. The new chlorophyll protein was discovered by one of us (Yakushiji) during his investigation on the isolation of cytochromes from various plant material[5]. On extracting the leaves of the weed, Chenopodium album, with the ordinary phosphate buffer and viewing the brownish crude extract with the hand spectroscope, an absorption band was discovered at 565 mμ. Attempts were then made to isolate the substance and it was finally obtained in purified form[5].

ISOLATION AND PURIFICATION

Since this particular chlorophyll protein is highly light-sensitive, care must be taken to carry out the following purification procedures in dim (green) light, to obtain the substance in its native state. The substance is extracted from the fresh (or deep-frozen) leaves of Chenopodium album by homogenation in 0.01 M disodium phosphate solution and concentrated by collecting the fraction precipitating between 0.3 and 0.6 saturation with ammonium sulfate. The precipitate is dissolved in 0.01 M phosphate buffer, pH 7.8 and, after dialysis against the same buffer solution, subjected to chromatography on a column of Amberlite CG 50. The substance can be adsorbed and washed on the column at concentrations of 0.01 - 0.02 M, and eluted out at 0.5 M of the phosphate buffer, pH 7.8. Subsequent ammonium sulfate fractionation was repeated several times and the final product of the pure chlorophyll protein was stored in 0.1 M phosphate buffer, pH 7.8, which may be stored without any deterioration, if kept in the dark and in a frozen state.

PROPERTIES OF CHLOROPHYLL PROTEIN

The substance is water-soluble and gives a clear solution when dissolved in water or buffer solution. The solubility and stability in plain aqueous media

(i.e., without addition of any detergent) are one of the remarkable characteristics of this particular chlorophyll protein. The ultracentrifugal analysis of the purified sample in a Spinco Ultracentrifuge (Model E, at 14° C) showed a single-peaked sedimentation pattern, indicating the homogeneous dispersion of the substance in the solution. The sedimentation constant was computed to be $S_{20'} = 2.7$.

Spectral Characteristic

Fresh solution of the chlorophyll protein prepared in the dark is green in color, which changes on standing in the light into yellowish-green. The solid line in Fig. 1 shows the absorption spectrum of the native chlorophyll protein.

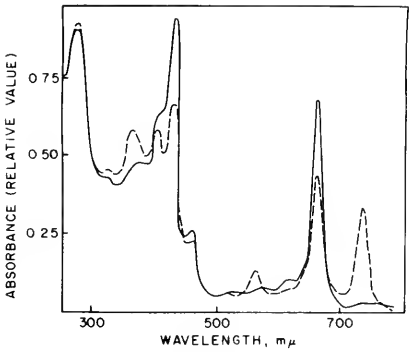

Fig. 1 (left) Absorption spectra of Chenopodium chlorophyll protein (CP 668; native form, CP 743; illuminated (partially photoconverted) form)

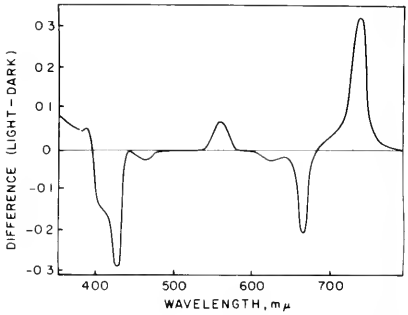

Fig. 2 (right) Difference spectrum of photoconversion of Chenopodium chlorophyll protein (CP 743 minus CP 668)

The main peak at 668 mμ corresponds to the red-band of chlorophyllous complex, the accompanying smaller accessory peak at 615 mμ, 575 mμ and 530 mμ are also common in this group of substance. Another main absorption at 430 mμ and the shoulder at 410 mμ represent the Soret bands of the chlorophyllous complex. The absorption at 277 mμ is probably due to the protein content. The dotted line in the same figure shows the absorption spectrum of the same sample of the chlorophyll protein, which had been illuminated for 30 seconds with white light (10,000 lux). A new far-red peak appears at 743 mμ, and there are also changes in the shorter region of the spectrum, new peaks appearing at 364 mμ and 400 mμ. The appearance of the peak at 565 mμ is also remarkable. The absorption at about 700 mμ also rises considerably, although it does not make a significant absorption peak. The difference spectrum of the light-induced changes is shown in Fig. 2.

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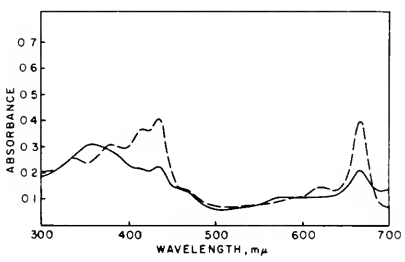
Pigment Components of the Chlorophyll Protein

On addition of higher concentrations of organic solvents, such as alcohol and acetone, there occur abrupt changes in absorption of the chlorophyll protein. The red band of the non-illuminated form shifts from 668 mμ to 663 mμ, and the far-red peak at 743 mμ characteristic of the illuminated form disappears by the treatment to give rise to a new but less remarkable peak at 718 mμ. With still higher concentrations of the organic solvent, the green color of the substance is extracted in solution. The absorption spectra of such alcohol extracts are shown in Fig. 3. The pigment in the extract was analyzed by paper chromatography according to Jeffrey, using petrol ether containing 2% isopropanol as developing solution(6). The presence of chlorophylls a and b in the extract of the non-illuminated sample was confirmed by spectrophotometric examination of the pigments eluted from the corresponding spots. In the chromatogram of the illuminated form, the spot corresponding to chlorophyll a considerably diminishes, while the spot of the b component remained essentially the same. These features correspond to the difference between the absorption spectra of the extracts of illuminated and non-illuminated samples of the chlorophyll protein. There were two new green spots also showing red fluorescence in the chromatogram of the illuminated form, which remain still unidentified. No carotenoid content was discovered in the purified substance. The protein nature of the substance was also confirmed by the ninhydrin and Biuret tests.



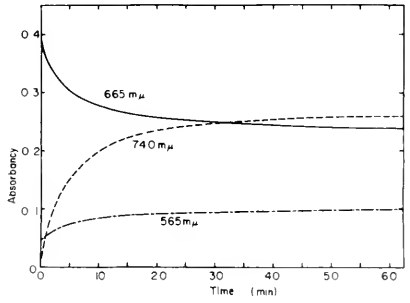


Fig. 3 (left) Absorption spectra of alcoholic extracts of Chenopodium chlorophyll protein (CP 668 and CP 743)

Fig. 4 (right) Time course of photoconversion of Chenopodium chlorophyll protein; CP 668 → CP 743

From the above-described facts and especially from the content of chlorophylls a and b in its native (i.e. non-illuminated) form, the substance was inferred to be a chlorophyll-protein compound and the designation as Chenopodium chlorophyll protein CP 668 is proposed according to the position of its red-peak. The illuminated form will be indicated as CP 743.

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Photoconversion of CP 668 to CP 743

Since the most prominent feature of this chlorophyll protein is its sensitivity towards light, experiments were carried out concerning the above-mentioned photoconversion of the dark form, CP 668, to the illuminated form, CP 743. The reaction was followed by measuring the main absorption changes at 565 mμ, 668 mμ and 743 mμ. It was confirmed by the preliminary experiments that there was a definite correlation between these photo-induced increases and decreases in absorbancy. Fig. 4 shows the time course of the photoconversion.

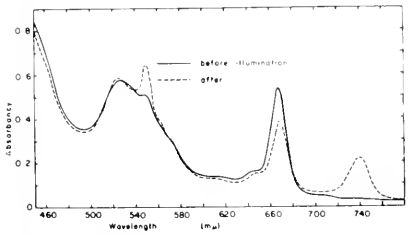
First, it was confirmed that the presence of air in the reaction mixture is a necessary condition for the photoconversion of CP 668. After removal of molecular oxygen from the solution, the chlorophyll protein was quite stable towards illumination. On introduction of oxygen (air) to the evacuated system, the capacity for the photochemical change was immediately recovered, the same spectral changes being readily observed on subsequent illumination (i.e., decrease of 668 mμ peak, accompanied by the appearance of peaks at 743 mμ and 565 mμ).

Oxidizing agents other than oxygen were then tested in this respect. Potassium ferricyanide at a concentration of 5×10^{-3} M was found to replace molecular oxygen. The rate of photoconversion in this case, however, was rather slower than the rate under the light-aerobic condition. Also with ferricyanide there was no reaction in the dark. The reaction in the presence of mammalian cytochrome c is shown in Fig. 5. As will be seen in the figure, the light (670 mμ, monochromatic illumination) induced the conversion of CP 668 to CP 743. The rise of the peak at 550 mμ is a result of simultaneous reduction of the cytochrome added (similar result was observed with algal cytochrome 553)[7]. Also in this case, there was no formation of CP 743 in the dark.

2, 6-Dichlorophenol indophenol (DPIP) also was effective in inducing the photoconversion. The time course of the reaction is shown in Fig. 6. The absorption increase at 565 mμ in this case, however, was not clearly detected owing to the absorption due to the dye added. There was no change in absorption in the dark.

Reversibility of Photoconversion

In view of the expected role of the substance in some oxidation-reduction reactions, the reversal of the above-described change of the chlorophyll protein was tested. Light or dark incubation of the illuminated form of the chlorophyll protein in a pure nitrogen atmosphere did not result in any absorption increase at 668 mμ or decrease at 743 mμ. Considering the circumstance that the presence of an oxidizing reagent made a requirement in the photoconversion, the effects of reducing substances were tested in reversing the change. Among various reducing agents tested, including sodium hydrosulfite, ascorbate, phenyl



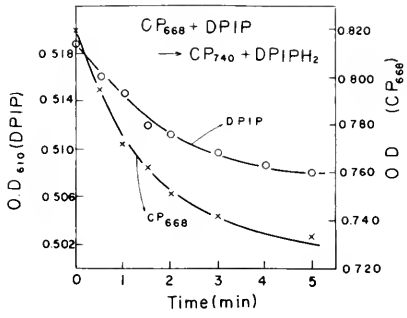


Fig. 5 (left) Photoconversion of Chenopodium chlorophyll protein in presence of mammalian cytochrome c

Fig. 6 (right) Photoconversion of Chenopodium chlorophyll protein 668 in presence of DPIP

hydrazin, EDTA, sodium borohydride, Pd with H_2, and methyl viologen, the first two named gave successful results with respect to the reversion of the spectral changes in question.

In an experiment shown in Fig. 7, the chlorophyll protein was first photoconverted into the illuminated form, CP 743, in the air (see above). After the removal of oxygen, the cuvette was sealed and illuminated for 30 minutes in the presence of added sodium hydrosulfite (10^{-3} M). About 97% of the CP 743 previously formed was re-converted into the initial form, CP 668. In this case, the marked decreases in absorbancy at 458 mμ and 645 mμ caused

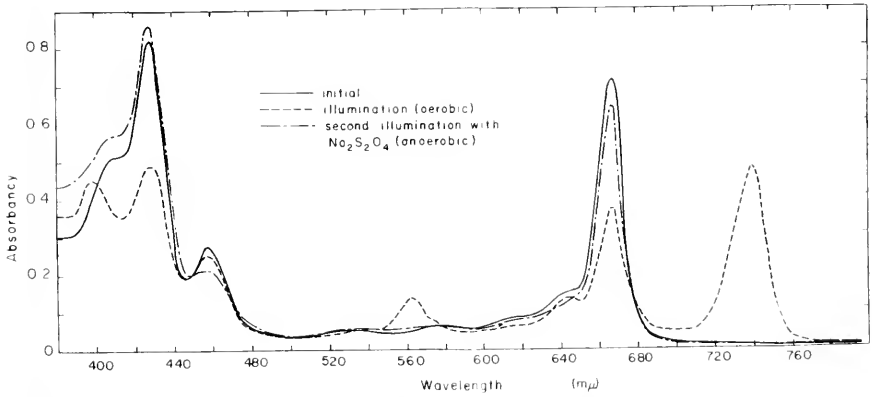


Fig. 7 Photoreversion of Chenopodium chlorophyll protein in presence of sodium hydrosulfite

by illumination remain unexplained. In the presence of added sodium hydrosulfite (anaerobic), there was a slow but detectable dark-conversion of CP 743 to CP 668, resulting in a partial recovery (about 60 - 70%) of the initial non-illuminated form in several hours of incubation. The back reaction-rate in the dark was, however, insignificant compared with that which occurred in the light.

Ascorbic acid was without effect in the dark. On illumination of CP 743 in its presence (alkaline phosphate solution, pH 12; anaerobic), however, there was an almost complete recovery of CP 668. At pH 7.8, the light-induced recovery was sluggish and rather inconsistent. It was noticed that, also in this case, the recovery of the peak at 458 mμ does not accompany the rise of the 668 mμ peak. The small shoulder at 645 mμ was also abolished during the incubation in the light in the presence of ascorbate.

Monochromatic light at 667 mμ, 430 mμ and 465 mμ were effective in inducing the photoconversion from CP 668 to CP 743, and light at 740 mμ and 565 mμ for the peak reaction. This fact suggests that these two sets of absorption peaks arise from the same substance, i.e., CP 668 and CP 740, respectively.

From the above-described facts concerning the interconversion between the dark and illuminated forms of the chlorophyll protein, it was inferred that the changes under investigation involve some oxidation-reduction reaction. This inference leads us to the tentative conclusion that the dark and illuminated forms represent the reduced and oxidized forms, respectively, of the chlorophyll protein.

The findings reported here may be of significance in view of the growing interest in far-red absorbing forms of chlorophyll. Similar but not identical substances have been discovered in various green organisms by French[8], Allen[9], Brown (10), Butler[11], and Lippincott[12]. On the other hand, an absorption change has been found by Kok[13] to occur at 710 mμ in the illuminated chloroplasts.

The question whether our chlorophyll protein is a product peculiar to Chenopodium album, or whether it occurs generally (when it may occur at lower concentration) and play some physiological role in photosynthesis must await further investigation.

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The authors' thanks are also due to Dr. H. Tamiya, Tokugawa Institute for Biological Research (Tokyo), Dr. K. Okunuki, Osaka University (Osaka) and Dr. Koidzumi, Tohoku University (Sendai), for their friendly support.

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STUDIES OF THE CONSTITUTION AND PHOTOCHEMICAL ACTIVITY OF AN ISOLATED CHLOROPHYLL COMPLEX

M. B. Allen and J. C. Murchio

This paper describes recent progress with investigations directed toward the isolation and characterization of chlorophyll complexes of living algal cells and study of the photochemical activity of the isolated complexes. We have previously reported (1,2) the isolation from Chlorella pyrenoidosa of a photochemically active chlorophyll complex with its red absorption maximum at 672 mμ (P-672). This complex has a chlorophyll a/b ratio of approximately 1, and also differs slightly from the bulk of the chloroplast material in its carotenoids. On illumination it forms a stable free radical that can be discharged by reaction with natural or artificial electron acceptors.

Under the best conditions so far achieved, the yield of P-672 is 1 to 1.5% of the total chlorophyll of the cell. Success in its preparation depends upon breaking the cells in the right manner to separate this particular fragment from the chloroplast, and upon centrifugation at the proper viscosity to effect its separation from other fragments. Even small variations in the cell breaking processes of grinding and ultrasonic treatment result either in no yield or in the formation of a so far inseparable mixture of products exhibiting a flat topped absorption curve with maximum at 675 mμ.

P-672 as obtained by previously described procedures was contaminated with a considerable amount of colorless material, some of it particulate, including RNA. Treatment with ribonuclease (RNAse), followed by washing, removed much of this material, as evidenced both by microscopic observation and by a decrease in absorbance in the 260-280 mμ region, with a concomitant increase in ultraviolet absorption in the washings. Treatment with desoxyribonuclease had no effect. Incubation with papain resulted in breakdown of the complex.

Among the noteworthy properties of P-672 is a tendency to form membranes whenever its concentration becomes great enough, be it through centrifugation, freezing, or slow dehydration with silica

gel. A typical membrane is shown in Figure 1. On formation of membranes the absorption maximum moves toward longer wavelengths, from 672-673 mμ to 677-678 mμ. Previous studies [3] indicate that it is unlikely that this change is a scattering artifact with our instrumentation. The membranes are astonishingly stable, vigorous ultrasonic treatment being required for their disintegration.

The implications of this spontaneous membrane formation for chloroplast structure remain to be explored. In the present investigation, it raised the possibility that the photochemical properties of the membranes might be different from those of the presumed particle originally split from the chloroplast. We have therefore been experimenting with techniques for purification and concentration of P-672 that do not involve pelleting the material. The most successful of these so far comprises passage through a column of Sephadex G-200 after RNAse treatment, followed by concentration with silica gel, stopping before the point of membrane formation. This treatment removes most of the material absorbing in the 260-280 mμ region, as well as whatever natural electron acceptors that react with the light induced free radical are present in the crude P-672 preparation. The purified material shows a distinct absorption peak at 340 mμ. The material responsible for this peak has not yet been identified.

Electron micrographs of material thus purified, prepared by the spray droplet technique of Williams and Backus [4], are shown in Figures 2, 3, and 4. The preparations contain particles of various sizes, the smallest being 70-75 Å, proceeding through 130-140 Å, 200-210 Å, to the largest, slightly greater than 300 Å. Small aggregates of these particles are also visible in the micrographs. The particle sizes observed suggest polymers of a fundamental unit of 70 Å or less. However, control experiments have shown that only about half of the particles in this preparation contain chlorophyll, the remainder probably being ribosomal contaminants. Until these can be removed it is not possible to be assured that all the size classes represent chloroplast particles.

For the same reason, it is only possible to give order of magnitude figures for the chlorophyll content and molecular weight of the particles. They contain 10-20% chlorophyll and have an average molecular weight of the order of 1,000,000. The results of papain treatment and extraction with fat solvents indicate a lipoprotein complex, as might be expected. The possibility of the presence of nucleic acids as an integral part of the complex is being explored, but cannot, obviously, be settled until the ribosomal contaminants are removed.

M. B. Allen and J. C. Murchio

The photochemical activity of the complex that has been most extensively studied is the formation of a free radical on illumination. This radical exhibits a single line electron spin resonance (ESR) signal of line width and g value similar to the signal induced by long wave length red light in intact cells of C. pyrenoidosa.[5,6] However, unlike the signal from the intact cells, which decays in a few milliseconds, that in P-672 persists upon darkening the cells [2]. The radical can, however, be discharged by reaction with ferrocyanide or with natural electron acceptors present in the soluble fraction of the cell homogenate. These properties, as well as the redox potential calculated from the degree of discharge of the radical in ferri-ferrocyanide mixtures, are strikingly similar to those described for P-700 by Kok, Beinert, and Hoch [7,8]. It has therefore been of interest to determine whether these properties are common to different kinds of chlorophyll complexes, or whether the P-700 system is contained in P-672.

Action spectra for induction of the ESR signal were obtained with a Varian 4500 EPR spectrometer with optical transmission cavity. Light from a 500 w. projection lamp was collimated with aspheric lenses and passed through narrow band Spectrolab interference filters into the cavity. The voltage on the light was varied so that equal numbers of quanta were supplied at all wavelengths except 400 and 435 mμ, where the lamp output was insufficient to obtain this. Potassium ferrocyanide was added to the P-672 preparations to discharge the radical after illumination.

Results of such measurements are shown in Figure 5, both for material containing membranes (pelleted) and for the dispersed particles. The former shows a distinct peak at 695 mμ, as well as in the blue, with smaller peaks in the central portion of the spectrum. The signal in this sample increased linearly with light, so that the peak in the blue is considered significant even though lower light intensities were used for these points. The dispersed material gives a rather featureless curve except for a small peak at 695 mμ and a large one in the blue. The 695 mμ peak has been observed with chlorophyll concentrations varying from 0.8 to 2.7 mg chlorophyll/ml.

The curves shown in Figure 5, like other ESR action spectra, show the steady state signal level as a function of wavelength. The interpretation of such curves is not clear, since this is not an action spectrum in the usual sense of the term, in which the rate of a reaction, e.g. oxygen evolution, is determined at different wavelengths. In order to obtain information that might

be more readily interpreted, the growth and decay of the signal (in the presence of ferrocyanide) were examined.

The rate of increase of signal in P-672 on illumination is much lower than that in the intact cell. In whole cells of C. pyrenoidosa ca. 50 millisec. is required for growth of the signal, whereas in P-672 the rise time is ca. 5 sec. with white light in membranous material and greater than this in dispersed particles. The rate of rise depends on wavelength, but is in all cases faster than the rate of reaction of the radical with ferrocyanide. The curve for rate of increase of signal as a function of wavelength for equal numbers of incident quanta is shown in Figure 6. The peak at 695 mμ clearly indicates that the long wavelength pigment system is responsible for the signal in these particles. The slow rise in the blue is particularly striking. Although the steady state signal level is large in this region of the spectrum, its rate of production is very low. The peak of activity in the green remains a puzzle, since none of the known photosynthetic pigments in green cells absorbs in this region. The reasons for the lack of activity of the short wave length pigment system in a particle that appears from spectroscopic data to be enriched in this system are being explored.

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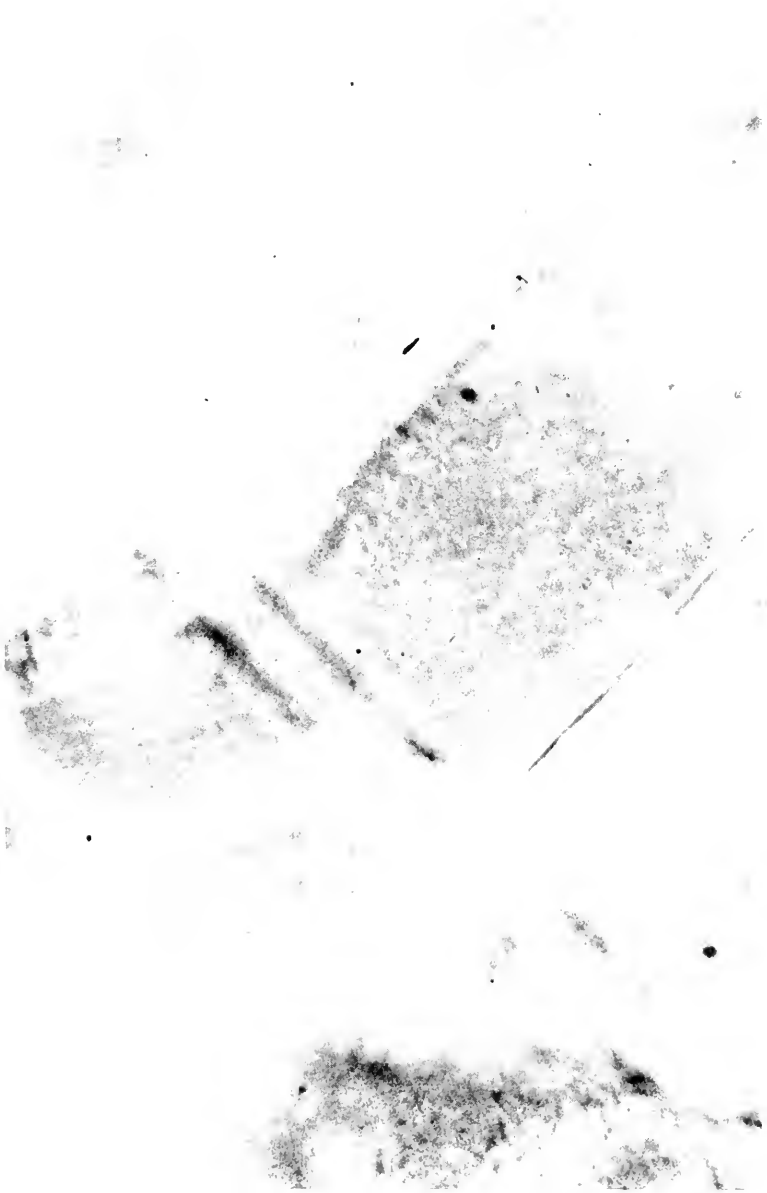

Fig. 1. Membranes formed on concentration of P-672.

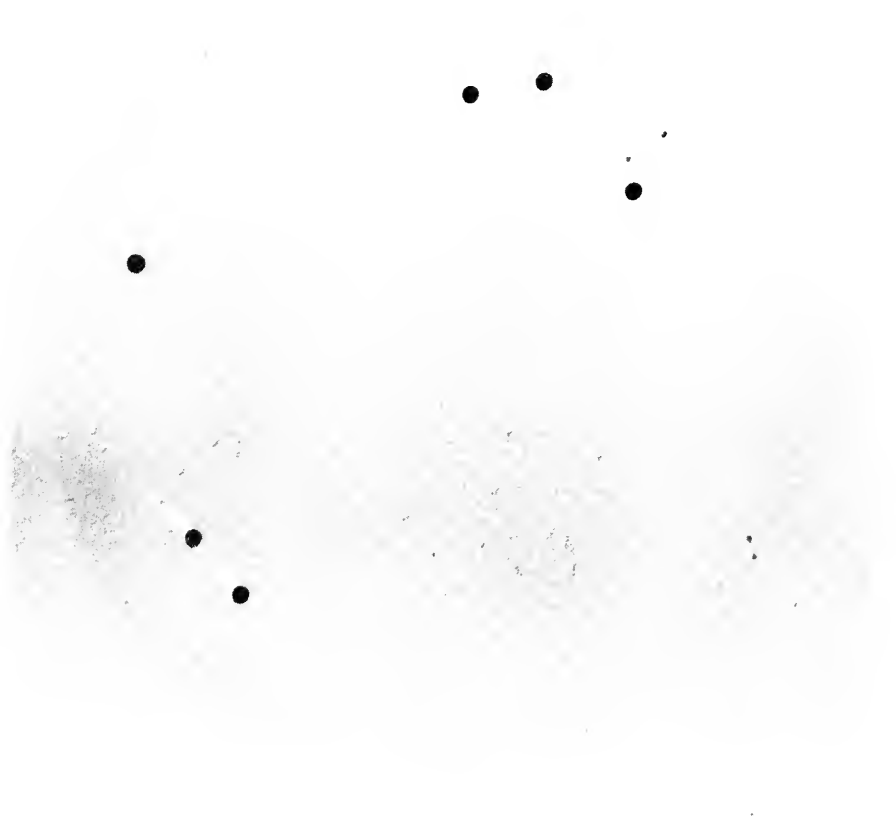

Fig. 2. Whole droplet, fixed in osmic acid, uranium shadowed. Magnification ca. 10,000.

Fig. 3. Part of droplet, fixed in osmic acid, uranium shadowed. Magnification ca. 30,000.

Fig. 4. Part of droplet, fixed in 10% formalin, uranium shadowed. Magnification ca. 100,000.

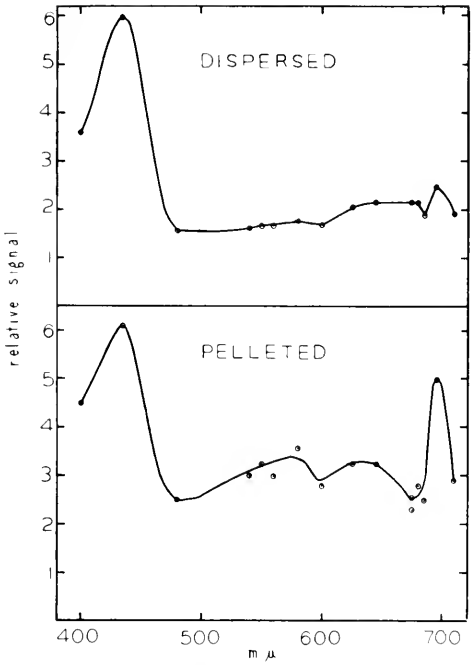

Fig. 5. Steady state levels of ESR signals in membranous and dispersed P-672 preparations as a function of the wavelength of light used for illumination.

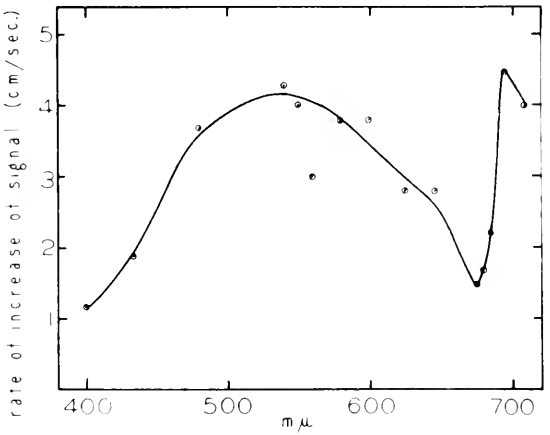

Fig. 6. Action spectrum for rate of formation of free radicals in a membranous preparation of P-672.

SOLUBLE PROTEIN-PIGMENT COMPLEXES FROM SPINACH CHLOROPLASTS

Joseph S. Kahn

In recent years there has been a growing interest in the isolation of subunits of chloroplasts containing portions of their photochemical and electron transport pathways. Such subunits could aid in elucidating the pathway of electron flow in the chloroplast, as well as provide a clue to the mechanism of the photochemical reaction itself. One of the most difficult aspects of this approach has been the isolation of photosynthetic pigments in conjunction with the proteins with which they are associated. The isolation of a soluble cytochrome c photooxidase, and of a protein-chlorophyll complex which could be bleached to produce three new pigments, have been reported (1,2). Also, a protein-β-carotene complex has been isolated from chloroplasts (3). Disruption of chloroplasts by sonication has yielded small fragments which could be the catalyst for one of the two light reactions required for photosynthesis (4).

This report describes two soluble protein-pigment complexes which were isolated and purified from spinach chloroplasts and which, in combination, catalyze the photoreduction of ferricyanide.

A Soluble Protein-Chlorophyll Complex

By fractional solubilization of chloroplast fragments with Triton x-100 and chromatography on DEAE-cellulose in the presence of Triton, a soluble protein-chlorophyll complex was isolated. Details of the techniques have been reported elsewhere (5). The complex represents 3-8% of the total chlorophyll of the chloroplast and contains 0.8-1.2 mg protein/μmole chlorophyll. The low-temperature spectrum of the complex reveals the chlorophyll to be chlorophyll a.

In order to avoid difficulties caused by chlorophyll bleaching, and in order to increase the sensitivity of the assay, ferricyanide reduction was measured with o-phenanthroline and the Fe^{+3} chelate of o-hydroxyethylene-diamine diphenyl acetate (Sequestrene 138 Fe of the Geigy Chemical Co.) (5). The protein-

chlorophyll complex was able to photoreduce ferricyanide but not di- or trichlorophenol-indophenol or NADP, the pH optimum for the ferricyanide reduction being 8.0-8.5, similar to that for chloroplasts. The reduction of ferricyanide appeared to be non-enzymatic, since the activity of the complex was not greatly reduced by boiling for 5 minutes. No photophosphorylation could be detected, but a slow photohydrolysis of ATP, amounting to 2-3 μmole/mg chlorophyll/hr, could be observed. Plastoquinone could not be detected in the protein-chlorophyll, and the addition of plastoquinone to protein-chlorophyll extracted with iso-octane had no effect on ferricyanide reduction (Table I). Menadione gave a small but consistent stimulation, the reason for which will become apparent later.

Table I

The effect of plastoquinone and menadione on ferricyanide reduction by the protein-chlorophyll. The plastoquinone and menadione were dissolved in ethanol to give a final concentration of 5% ethanol in the reaction mixture.

	μmole ferricyanide reduced / mg chlorophyll x hr	% of control
Control	23.1	100
Extracted with isooctane	22.1	96
+ 5×10^{-5} M plastoquinone	21.4	93
+ 5×10^{-4} M menadione	27.7	120
+ 5% ethanol	23.4	101

Polarographic measurements failed to show any evolution of oxygen during ferricyanide reduction. In agreement with this, CMU (p-chlorophenyl-1,1-dimethyl urea), which inhibits oxygen evolution in whole chloroplasts and thus inhibits their ferricyanide reduction, failed to decrease the activity of the protein-chlorophyll. Although there was a substantial bleaching of the chlorophyll during illumination, there was no stoichiometry between the chlorophyll bleached and the ferricyanide reduced. The nature of the electron donor for the reduction is as yet unknown.

When the protein-chlorophyll was treated with reduced thiols and then dialyzed under a nitrogen atmosphere, ferricyanide reduction was strongly inhibited. The inhibition appeared to be due to the formation of -SH groups rather than the disruption of disulfide bonds, since the inhibition could be reversed by p-chloromercuribenzoate (Table II). Arsenite or thorough aeration also reversed the inhibition by reduced thiols.

Table II

The effect of reduced thiols and of p-chloromercuribenzoate (PCMB) on the rate of ferricyanide reduction by the protein-chlorophyll.

The thiols were dialyzed out before assay. PCMB was added to the reaction mixture.

	μmole ferricyanide reduced / mg chlorophyll x hr	% of control
control	23.1	100
5×10^{-4} M PCMB	20.0	87
5×10^{-4} M glutathione (red.)	7.8	34
5×10^{-4} M cysteine	7.1	31
5×10^{-4} M β-mercaptoethanol	7.3	32
glutathione + PCMB	19.6	85
cysteine + PCMB	22.7	98
β-mercaptoethanol + PCMB	18.4	80

The rate of ferricyanide reduction by the protein-chlorophyll as a function of light intensity is given in Figure I. Maximal activity appears to be reached at 7000-8000 foot-candle, but measurements at these light intensities were uncertain because of the rapid bleaching of the chlorophyll. The rate of ferricyanide reduction was linear only up to 10 μgm chlorophyll/ml of the reaction mixture; moreover, the rate decreased during illumination even at low light intensities, becoming non-linear after 5-10 minutes. As a consequence, the activity below 30

foot-candle was too low to be reliably measured by our assay method.

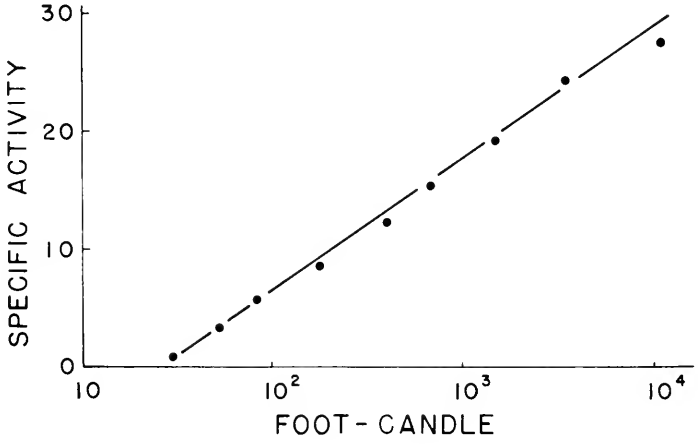

Figure I. The rate of ferricyanide reduction by the protein-chlorophyll as a function of light intensity.

A Water Soluble Protein-Carotene Complex

While attempting to separate the protein-chlorophyll into the protein and chlorophyll moieties, we succeeded in isolating a second protein-pigment complex from the protein-chlorophyll (6).

Samples of the protein-chlorophyll were extracted 5 times with equal volumes of 25% diethyl ether in petroleum ether. The pooled solvent was washed twice with water, dried, dissolved in 0.002 M Tris buffer, pH 8.0 and cleared by centrifugation. The resultant yellow solution was slightly opalescent.

The absorption spectra of the extracted protein-chlorophyll and of the extract in buffer are given in Figure II. The absorption maxima of the extract in different solvents are presented in Table III. No quinone derivatives reducible with

borohydride could be detected in the extract.

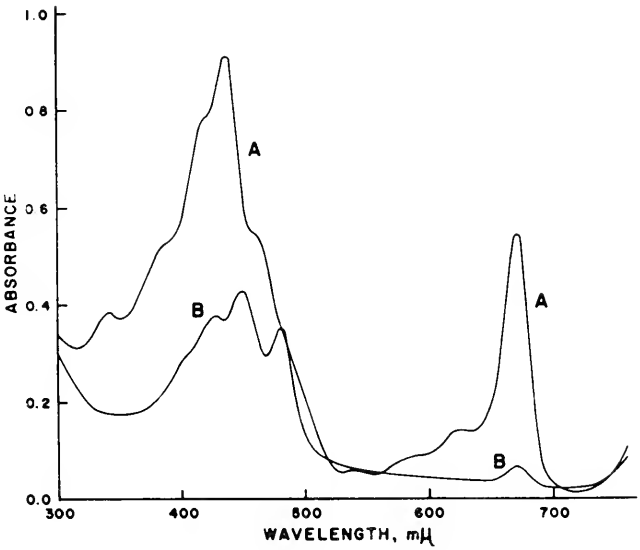

Figure II. Absorption spectra of protein-chlorophyll extracted with 25% diethyl ether in petroleum ether, (A) and of the extract dissolved in water (B).

Table III

Absorption maxima of the yellow pigment extracted from protein-chlorophyll, dissolved in various solvents.

Solvent	Wavelength, mμ		
water	426	451	481
petroleum ether	416	435	468
chloroform	427	449	480
carbon disulfide	442	468	500

Samples of the extract were dried, dissolved in n-heptane and chromatographed on aluminum oxide, powdered sugar, and starch by thin-layer chromatography. On the basis of the absorption spectra and the chromatographic behavior the pigment was identified as a carotenoid, probably a carotene.

The carotene-containing extract was further purified by chromatography on DEAE-cellulose, all the carotene appearing in a single peak containing 8.6-10.8 mg protein/μmole carotene. This protein was firmly bound to the carotene and could be separated only in part by extraction with dry solvents.

The molar ratio of chlorophyll to carotene in the crude protein-chlorophyll varied from 7 to 14. The protein-chlorophyll from which the protein-carotene had been extracted lost its ability to reduce ferricyanide, but the activity could be restored by adding back the protein-carotene, the optimal molar ratio of chlorophyll to carotene being about 10. When added to whole or broken chloroplasts, the protein-carotene completely inhibited ferricyanide reduction. Plastoquinone, β-carotene and FMN were unable to reactivate the protein-chlorophyll, but menadione could substitute for the protein-carotene. The optimal concentration for menadione was, however, 1000 times higher than that of the protein-carotene. The data are summarized in Table IV. This effect of menadione will explain its consistent small stimulation of ferricyanide reduction in crude protein-chlorophyll (Table I), since most preparations had less than the optimal concentration of protein-carotene.

The protein-carotene was not changed during ferricyanide reduction, and could be recovered intact after total bleaching of the chlorophyll. No stoichiometry existed between the amount of protein-carotene present and the amount of chlorophyll bleached.

In conclusion, we have isolated soluble protein-chlorophyll and protein-carotene complexes from spinach chloroplasts which together are able to photoreduce ferricyanide. In light of the fact that no oxygen was evolved during the reduction of ferricyanide, and that CMU had no inhibitory effect, these complexes may represent a model for that photochemical reaction in photosynthesis, involved in raising the potential of the reductant enough to reduce NADP.

The protein moiety in both complexes appears to have a non-enzymatic function in their activity, and may be the factor

which makes the pigments preferentially water-soluble. In the case of the carotene, all activity is lost when the pigment and protein are separated; in the case of the chlorophyll, we have been unable as yet to achieve a complete separation.

We do not know as yet whether these complexes represent a unique fraction or whether they are representative of most or all the chlorophyll in the chloroplast. However, we would like to believe that the reduction of ferricyanide by the combination of the two soluble complexes, is by a mechanism similar to that catalyzing the reduction by whole chloroplasts. If so, it may represent a useful tool for the study of the photochemical reactions involved in photosynthesis.

Table IV

Reactivation of ferricyanide reduction by protein-chlorophyll extracted with 25% diethyl ether in petroleum ether.

	μmole ferricyanide reduced / mg chlorophyll x hr	% of control
Unextracted protein-chlorophyll	31.5	100
+ protein-carotene (1:10)*	36.2	115
Extracted protein-chlorophyll	6.9	22
+ 10^{-6} M β-carotene (1:10)*	6.7	21
+ 5 x 10^{-5} M plastoquinone	7.3	23
+ 10^{-4} M FMN	10.1	32
+ protein-carotene (1:10)*	35.4	112
+ 10^{-3} M menadione	38.4	122
+ protein-carotene and menadione	49.6	157

*Approximate molar ratio of carotene to chlorophyll.

Acknowledgment

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PHOTOREDUCTION OF NADP BY ASCORBATE AND HEMATOPORPHYRIN

Anthony San Pietro, Leo P. Vernon and Dorothy Limbach

Some years ago, Krasnovsky demonstrated a photochemical reduction of chlorophyll by ascorbic acid in pyridine solution [1]. It was shown further that chlorophylls in light catalyzed the transfer of electrons from ascorbate to a wide variety of compounds including the pyridine nucleotides [2-4]. Whereas most of the reactions studied by the Krasnovsky group have been carried out in non-aqueous media, certain of them did occur in aqueous media [5].

Vernon [6] extended these observations and showed that the photoreduction of pyridine nucleotides in the presence of ascorbate and partially purified photosynthetic pyridine nucleotide reductase (PPNR) is catalyzed by chlorophyll a, chlorophyll b, hematoporphyrin, protoporphyrin and coproporphyrin. The significance of this finding is that the reaction occurs in aqueous solution and requires the protein PPNR which functions in the natural photosynthetic process wherein pyridine nucleotides are reduced [7].

During the course of the purification of PPNR it was observed that the reduction of pyridine nucleotides by illuminated chloroplasts requires a flavoprotein [8, 9], pyridine nucleotide transhydrogenase (transhydrogenase), in addition to the purified PPNR. There was no reduction of pyridine nucleotides in the presence of either protein alone; when both proteins were present, the reduction of pyridine nucleotides was observed.

In his initial experiments Vernon [6] used a partially purified preparation of PPNR which is known also to contain the transhydrogenase. It was of interest, therefore, to determine whether the hematoporphyrin and ascorbate system also requires both proteins as does the chloroplast system. Contrary to expectation, it was found that the reduction of pyridine nucleotides by ascorbate and hematoporphyrin requires only the transhydrogenase. Purified PPNR was completely inactive when present either alone or together with the transhydrogenase. Although NADP was used as the electron acceptor in the experiments reported, similar results were obtained with NAD.

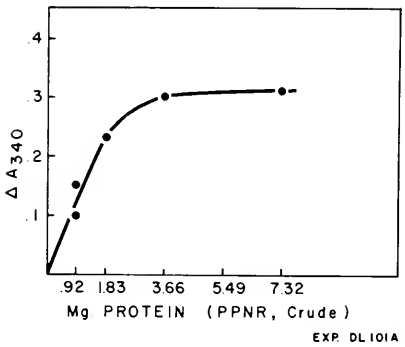
EXPERIMENTAL

The experimental procedures employed and the description of most of the materials used have been previously described [6, 9, 10]. In brief, the standard reaction mixture contained 0.21-0.27 μmole of hematoporphyrin; 144 μmoles of tris buffer, pH 7.8; 44 μmoles of sodium ascorbate; 2.7 to 3.4

μmoles of NADP and the indicated additions in a final volume of 8 ml. The time of illumination was 5 minutes.

RESULTS

The relationship between the rate of NADP photoreduction and the concentration of partially purified PPNR is shown in Fig. 1. At low protein concentration, there is a linear relationship between the rate of photoreduction of NADP and protein concentration; at higher protein concentrations, the rate is independent of protein concentration. This is not unreasonable since the experiments were not carried out at saturating light intensity. In separate experiments it was established that the rate of photoreduction of NADP was dependent on NADP concentration.



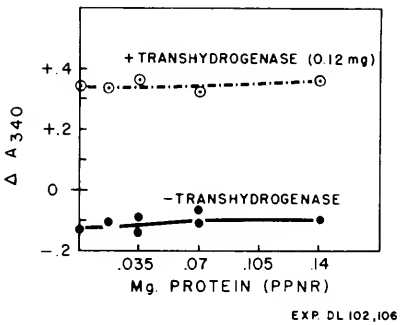
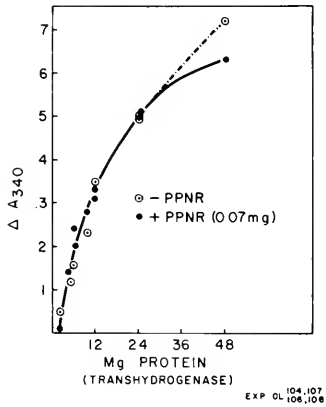


Fig. 1. (Left) Effect of Partially Purified PPNR upon NADP Reduction with Hematoporphyrin and Ascorbate.
Fig. 2. (Right) Lack of Effect of PPNR upon NADP Reduction with Hematoporphyrin and Ascorbate.

Contrary to the results obtained with the partially purified PPNR, the purified PPNR was completely inactive in catalyzing the photoreduction of NADP or NAD in this system (Fig. 2, Lower Curve). As can be seen there is a photobleaching of the hematoporphyrin in the presence or absence of purified PPNR (Fig. 2, Lower Curve). When transhydrogenase is included in the reaction mixture, there is observed a photoreduction of NADP (Fig. 2, Upper Curve). Furthermore, the rate of photoreduction of NADP in the presence of transhydrogenase is unaffected by the presence or absence of purified PPNR (Fig. 2, Upper Curve).

The relationship between the rate of photoreduction of NADP and the concentration of transhydrogenase is shown in Fig. 3. It is seen that there is a

linear relationship at low transhydrogenase concentrations. At higher transhydrogenase concentrations, there is some deviation from linearity. The same results are obtained whether or not the purified PPNR is included in the reaction mixture.



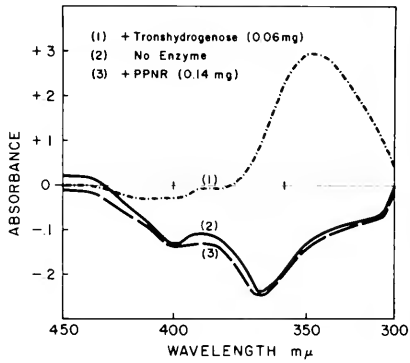


Fig. 3. (Left) Effect of transhydrogenase upon NADP Reduction with Hematoporphyrin and Ascorbate.
Fig. 4. (Right) Light Minus Dark Difference Spectra.

It can be seen that the curves presented in Fig. 3 do not go through the origin. This is due to the fact that there is a photobleaching of the hematoporphyrin when illuminated alone or in the presence of purified PPNR. Light minus dark difference absorption spectra for various reaction mixtures are presented in Fig. 4. When transhydrogenase and hematoporphyrin are both present, an increase in absorbance at 340 mμ is observed (Curve 1). For hematoporphyrin alone (Curve 2) or hematoporphyrin plus purified PPNR (Curve 3), there is a decrease in absorbance at 340 mμ upon illumination.

In contrast to most photosynthetic reactions which exhibit light saturation at about 1000 foot-candles, this reaction did not exhibit light saturation over the range of light intensities studied.

DISCUSSION

The photochemical reduction of pyridine nucleotides by chloroplasts is thought to require both PPNR and a specific flavoprotein [8, 11, 12]. It is generally assumed that the PPNR is reduced by the light-trapping system (chlorophyll a) and that the flavoprotein catalyzes the transfer of electrons from reduced PPNR to NADP.

In the experiments reported herein, the hematoporphyrin serves as the

light-trapping system and ascorbate as the hydrogen donor. If one assumes that this system functions in a manner analogous to the chloroplast system, then the transhydrogenase must catalyze a transfer of electrons from an activated form of hematoporphyrin to NADP. It would thus appear that in this soluble system transhydrogenase can interact directly with the activated hematoporphyrin without the intervention of PPNR. In the chloroplast system there is no direct interaction of the transhydrogenase and activated chlorophyll; the interaction between them is mediated by PPNR. The difference between these two systems is most probably due to the fact that one is a soluble system and the other is a particulate system with a very high degree of organization.

Recently, Smillie [13] has reported the isolation of a flavoprotein from Anacystis nidulans which exhibits PPNR activity. This flavoprotein will substitute for either spinach or Anacystis PPNR in a number of reactions which were thought to be specific for PPNR. There is some similarity between the data presented here and that reported by Smillie [13] but any possible significant relationship between them must await further experimentation.

It should be noted that the hematoporphyrin system exhibits specificity for the transhydrogenase flavoprotein as does the chloroplast system. Lazzarini and San Pietro [14] have isolated another flavoprotein from spinach which exhibits TPNH-diaphorase activity but no transhydrogenase activity. This flavoprotein did not substitute for the transhydrogenase flavoprotein in this system.

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This is Contribution No. 130 from the Charles F. Kettering Research Laboratory.

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PHOTOOXIDATION OF REDUCED PMS BY CHLOROPLASTS AND CHLOROPHYLL A UNDER ANAEROBIC CONDITIONS IN THE PRESENCE OF QUINONES

Leo P. Vernon, Waldo S. Zaugg and Elwood Shaw

INTRODUCTION

It is currently held that photosynthesis in green plants comprises two separate light reactions which couple through enzymatic components to complete the electron transfer span from water to reduced pyridine nucleotide. The primary photochemical act for the long wavelength system results in transfer of electrons from a cytochrome situated at the reaction center to an electron acceptor such as ferredoxin, which can then couple chemically with other electron acceptors such as NADP [1-4]. The long wavelength system in plant photosynthesis resembles in many respects the photosynthetic apparatus found in bacteria [4-7].

The photosynthetic process in bacteria involves the oxidation of cytochrome coupled to the reduction of some endogenous component within the chromatophore fragment, such as ubiquinone [8-10]. In studying this reaction in detail, Zaugg demonstrated that bacterial chromatophore fragments catalyze the photooxidation of added cytochrome c coupled to the photoreduction of added UQ_2 or UQ_6 under anaerobic conditions [11]. $PMSH_2$ can substitute for cytochrome c in this reaction, becoming oxidized in a coupled reaction with UQ_2.

Similar experiments have been attempted with chloroplasts. With detergents present and under aerobic conditions it was possible to demonstrate the photooxidation of ferrocytochrome c previously reported [12, 13]. It was not possible to demonstrate a cytochrome oxidation coupled with quinone reduction under anaerobic conditions. When PMS was substituted for cytochrome c, however, a coupled oxidation of $PMSH_2$ and reduction of UQ_2 was demonstrated with spinach or poke weed chloroplasts. UQ_6 was less active than UQ_2 in these reactions, but a marked stimulation was obtained upon the addition of the detergent Triton X-100. With this detergent present either UQ_2 or UQ_6 supported the photooxidation of $PMSH_2$. A rapid back reaction was observed in a subsequent dark period with both quinones. Chlorophyll a in the presence of Triton X-100 also catalyzes the reaction. Since this reaction is representative of the primary photochemical reaction thought to occur in the long wavelength system, it has been investigated in some detail.

Leo P. Vernon, Waldo S. Zaugg and Elwood Shaw

METHODS AND MATERIALS

Chloroplasts were prepared from either spinach or poke weed (Phytolacca americana) as previously described [5], and were stored in a frozen condition for several months with no appreciable loss of activity for the present reaction. Chlorophyll a was prepared by chromatography on a powdered sucrose column as described previously [14]. PMS was purchased from Sigma Chemical Company, UQ_6 and sodium deoxycholate from Mann Research Laboratories, and UQ_2 was a gift from Merck, Sharp and Dohme Research Laboratories. Triton X-100 (alkyl phenoxy polyethoxy ethanol) was purchased from Rohm and Haas, Philadelphia. Experiments were performed under anaerobic conditions with Thunberg tubes attached to either one or two absorption cells as previously described [9]. Anaerobic conditions were obtained by alternate evacuation and flushing with Argon gas [9]. The reaction mixtures were illuminated through a red filter (Corning No. 2403) by means of a tungsten filament lamp [9]. Absorbancy changes caused by illumination were determined with either a modified Bausch and Lomb 505 spectrophotometer [9] or a Beckman DB recording spectrophotometer [11].

RESULTS

The ability of chloroplasts to catalyze a coupled photooxidation of $PMSH_2$ and photoreduction of added UQ_2 is shown in Figure 1. For these experiments PMS was added initially in the oxidized form and the quinone in the reduced form, resulting in chemical reduction of PMS by the added reduced quinone before illumination was initiated. Upon illumination a rapid increase in absorbancy at 388 mμ was observed, indicating oxidation of the added $PMSH_2$. Control experiments in which reduced quinone was omitted and PMS

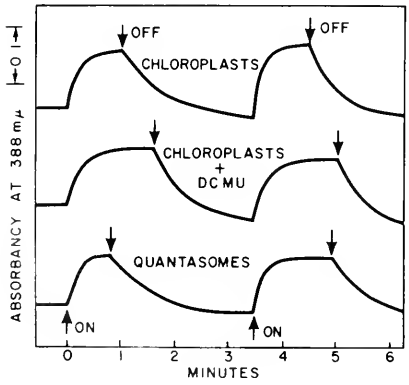
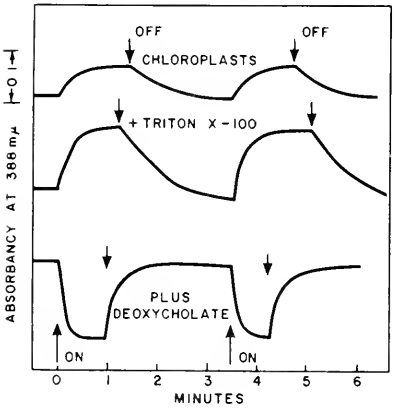


Fig. 1. Photooxidation of $PMSH_2$ by poke weed chloroplasts in the presence of UQ_2. Prior to illumination PMS was added in the oxidized form and UQ_2 was added in the reduced form (reduced by sodium borohydride). The reaction mixture was allowed to stand for 5 minutes during which time the PMS was reduced chemically by UQ_2H_2. Anaerobic conditions were employed. The reaction system contained in a final volume of 2.0 ml the following in μmoles: PMS, 0.1; UQ_2H_2, 0.2; phosphate buffer pH 6.6, 100; and DCMU (where indicated), 0.04. The chlorophyll content of the chloroplasts was 6 μgrams, and the light intensity was 358 milliwatts.

was reduced photochemically by white light in the absence of air showed that the quinone must be present for the photooxidation of $PMSH_2$ to proceed. Turning off the light allowed a back reaction to be observed, in which PMS was rereduced by the reduced quinone to give the original equilibrium concentration. The addition of DCMU, a specific inhibitor for photosynthetic oxygen evolution [15], had no effect upon the photoreaction. This was expected since this reaction should not involve the entire photosynthetic machinery, but only the long wavelength system. Quantasomes from poke weed were prepared according to the directions of Park and Pon [16] and were found to be active. This indicates that the entire chloroplast fragment is not required for this reaction, but only the primary photosynthetic system involved in photosynthetic electron transfer reactions.

The effect of detergents upon the photoreaction is shown in Figure 2.



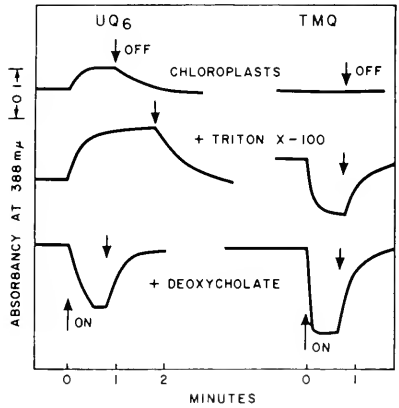


Fig. 2. (Left) PMS photoreactions in the presence of spinach chloroplasts, UQ_2 and detergents. Conditions as for Figure 1 were used, and where present Triton X-100 was 0.02 per cent and deoxycholate was 0.4 per cent.
Fig. 3. (Right) Photoreactions of PMS with poke weed chloroplasts and UQ_6 or TMQ (trimethylbenzoquinone). Reaction conditions as for Figure 1.

Triton X-100 at a concentration of 0.02 per cent markedly stimulated the rate of $PMSH_2$ photooxidation. The addition of deoxycholate at a final concentration of 0.4 per cent had just the reverse effect, influencing the reaction system to cause a photoreduction of PMS. It should be noted that the PMS was initially reduced by adding UQ_2 in the reduced form, allowing the UQ_2H_2 to react with PMS to an equilibrium position. Sufficient oxidized PMS remains to allow for a net photoreduction in the presence of deoxycholate.

Figure 3 shows that UQ_6 in the absence of added detergents is unable to

support an extensive photooxidation of $PMSH_2$. Addition of Triton X-100 allows the photooxidation to proceed, while addition of deoxycholate causes a photoreduction of PMS. The larger aliphatic side chain on UQ_6 decreases the solubility of this quinone in water. This may explain the inactivity of the UQ_6 in the absence of detergent.

TMQ, trimethylbenzoquinone, was also tested in photoreactions with PMS. This quinone was added in the reduced form to PMS and sufficient time allowed for the two compounds to react chemically. Subsequent illumination in the presence of chloroplasts caused no appreciable reaction. The addition of Triton X-100 resulted in a photoreduction of PMS, and a faster photoreduction rate was obtained by the addition of deoxycholate.

Although not examined in detail, other detergents were also tested as to their effect on the PMS photoreactions. Sodium lauryl sulfate, an anionic detergent, resembled deoxycholate in causing the chloroplasts to affect a photoreduction of PMS, while digitonin had very little effect. A cationic detergent ethylhexadecyldimethylammonium bromide also behaved like Triton, resulting in a marked stimulation of the photooxidation rate for $PMSH_2$.

A number of people have reported on the effect detergents have on chloroplasts and chloroplast activity. Solubilization of chloroplasts was accomplished in 1941 by Smith, using digitonin, bile salts, sodium deoxycholate and sodium dodecyl sulfate [17-19]. The first three detergents remove and solubilize the chlorophyll while the latter splits the chloroplast into protein units containing the pigment. Ke and Clendenning [20], using such detergents as sodium dodecyl sulfate, Zephiran chloride, Tween 20, Triton X-100, digitonin, bile salts and saponin, studied the effect these detergents had upon the Hill reaction and physical properties of the chloroplast. Eversole and Wolken prepared a pigment-protein complex called chloroplastin by treating chloroplasts with digitonin [21] and reported that it retained some of the photochemical activities of the chloroplasts. Nieman and Vennesland [12] reported that digitonin treated chloroplasts catalyze a photooxidation of ferrocytochrome c. Hinkson and Vernon [22] studied the effect of digitonin upon ascorbate photooxidation in the presence of DPIP, showing that digitonin treated chloroplasts are more than six times as reactive. Chiba and Okayama [23] studied the effects of Dupanol C and span 80 upon the photooxidations catalyzed by chloroplasts. These detergents solubilize the chloroplast and cause a marked increase in the photooxidation of ascorbate in the presence of DPIP under aerobic conditions. Kondo et al. [24] have studied the effect of surface active agents upon chloroplasts showing that cationic and anionic detergents have the effect of either fluculating or lysing (solubilizing) chloroplast preparations. They reported that nonionic detergents had no effect.

We have tested the effect of Triton X-100 concentration upon the chloroplast preparations used in our experiments. Table 1 shows that Triton X-100 is able to remove chlorophyll from the chloroplast with complete removal being obtained at a concentration of 0.02 per cent. Under these conditions

TABLE 1

Removal of Chlorophyll from Chloroplasts by Triton X-100

Chloroplasts equivalent to (a) 135 or (b) 27 ugrams of chlorophyll were extracted for 30 minutes with 10 ml of solution containing detergent at the indicated concentration. The mixture was then centrifuged for 30 minutes at 140,000 x g in the Spinco ultracentrifuge. The chlorophyll remaining in solution was determined from the absorbancy at 667 mμ.

Per cent Triton X-100	Per cent Chlorophyll Removed	
	a	b
.002	3	7
.005	11	53
.010	36	65
.020	53	100
.040	89	100
.080	100	100

the chlorophyll becomes solubilized and the preparation becomes optically clear. The physical properties of such solubilized chlorophyll have not been determined, so it is not known if it exists as free chlorophyll or is attached to a small particle. (Chiba [25] has reported the solubilization of a chlorophyll protein from chloroplasts through the use of Dupanol.) The absorption spectra of an original chloroplast suspension and the detergent-extracted material were determined with the Cary Model 14 spectrophotometer equipped with an apparatus designed for measuring absorption spectra from highly scattering suspensions. In the red region the absorption maximum of the chloroplast suspension occurred at 676 mμ. In the Triton X-100 extract this peak shifted to 667 mμ and was sharper and more intense. A sharpening of the absorption bands was also observed in the blue region. This agrees with the data presented by Kondo et al. [24] who examined the effect of various sodium alkyl sulfates upon the absorption spectra of chloroplasts and derived fractions.

The rates of $PMSH_2$ photooxidation under the various conditions of detergent and acceptor quinone are shown in Table 2. The reactions are shown as either a photooxidation (with ubiquinone and Triton X-100) or a photoreduction (with TMQ regardless of the detergent). The rates are the observed initial reaction rates obtained with the Spectronic 505 spectrophotometer. Since this instrument is limited in its response time, it is probable that the actual reaction rates are faster than those reported in Table 2.

TABLE 2

Rates of PMS Photoreactions with Spinach Chloroplasts

The reaction conditions were those given for Figures 1 to 3 for the appropriate quinones.

Quinone	Per cent Triton X-100	Per cent Deoxycholate	Rate of Photoreaction µmoles/hr/mg Chloroplasts	
			Photooxidation	Photoreduction
UQ_2	0	--	49	--
	.02	--	160	--
	.06	--	600	--
	.06	0.4	--	450
UQ_6	0	--	0	--
	0.4	--	620	--
	0.4	0.4	--	790
TMQ	0.04	--	240	--
	0.04	0.33	--	1740

The stimulation of the PMS photoreactions by the presence of Triton X-100 caused us to investigate the activity of isolated chlorophyll a. As shown in Figure 4, chlorophyll a in the presence of Triton X-100 has the

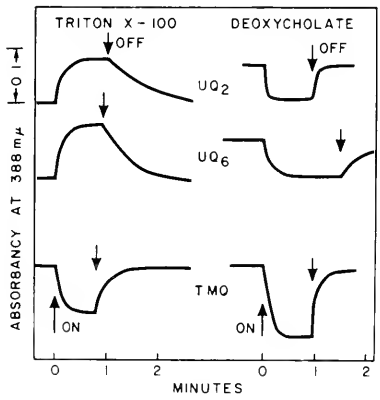


Fig. 4. Sensitization of PMS photoreactions by chlorophyll a in the presence of detergents. Chlorophyll a was added to the reaction mixture from a methanol solution to a final concentration of 4 µgrams. Other conditions as in Figures 1-3.

ability to catalyze the same photoreactions as does the intact chloroplast or the chloroplast in the presence of detergent. Thus, both UQ_2 and UQ_6 support a photooxidation of $PMSH_2$ while TMQ supports a photoreduction of the dye. In the presence of deoxycholate all three quinones support the photoreduction of PMS. For these experiments chlorophyll a was added to the detergent solution from a methanol solution. The resultant preparations were optically clear, and were carried through the manipulations in the ordinary fashion.

Although other porphyrins have not been investigated in as much detail, it should be mentioned that this reaction is not restricted to chlorophyll a. In the presence of Triton X-100 the following porphyrins support a photooxidation of $PMSH_2$ in the presence of UQ_2: chlorophyll a, chlorophyll b, bacteriochlorophyll, pheophytin a, chlorophyllin a, protoporphyrin IX, and tetraphenylporphyrin. It is interesting that the water soluble porphyrin, hematoporphyrin, is inactive. This is unusual since this compound has been shown to catalyze the photoreduction of methyl red, tetrazolium blue, and NADP in the presence of ascorbate [26, 27].

Under the experimental conditions employed the reaction was not saturated with respect to light intensity. This agrees with the fact that PMS stimulated photophosphorylation does not show the usual light saturation response [28]. A preliminary study of the effect of pH upon the reaction revealed that the nature of the reaction was sensitive to the pH of the medium. Thus, with UQ_6 at pH values between 6.6 and 7.6 the usual photooxidation of $PMSH_2$ was observed in Triton X-100 solutions. When the pH was lowered to 6.0, however, the reaction changed to one of photoreduction of PMS. This again indicates that the reaction is very sensitive to the ionic species in the reaction mixture.

DISCUSSION

PMS has played a singular role in the biochemical investigations of photophosphorylation and associated electron transfer reactions in both plants and bacteria. In the plant system PMS effectively catalyzes the process of photophosphorylation [4, 6, 28] by virtue of its being alternately oxidized and reduced, thus completing the cycle of electron flow driven by the photochemical system. Evidence for direct interaction of PMS with the components of the photosynthetic apparatus is presented from the extensive experiments of Witt and collaborators as summarized in reference 3. These data show that PMS can be oxidized either through the endogenous cytochrome (cytochrome f) or by chlorophyll a directly. The compound also operates in the bacterial system to catalyze cyclic electron flow in the presence of the inhibitor antimycin A, which inhibits one step of the cyclic electron transfer system. PMS serves as a bypass for this sensitive site, thus completing the cycle and allowing cyclic electron flow and photophosphorylation to proceed [29, 30].

The behavior of PMS in the photophosphorylation system is unique in its

response to light intensity. Whereas the FMN and vitamin K_3 reactions saturate at a moderate light intensity (20,000 lux), the PMS supported reaction does not saturate at light intensities of 50,000 lux or at 154,000 lux [28]. At these high light intensities the photophosphorylation rates obtained with PMS are high. This response indicates that PMS couples to the photochemical system in a unique manner, and furthermore that the photochemical reaction is the rate limiting step at all intensities employed in the PMS catalyzed photophosphorylation system. This is born out by the experiments of Hall and Arnon who investigated the effect of temperature upon various photophosphorylation systems at low light intensity [32]. Whereas vitamin K_3 and FMN stimulated photophosphorylation responded in the usual manner to temperature changes from -10 to 15° C, the PMS supported photophosphorylation did not change through this temperature range. This response was to be expected, since the photochemical reaction is limiting under these conditions.

The present investigation shows that PMS does indeed react directly with either the chloroplast or isolated chlorophyll a in the presence or absence of detergents. Furthermore, this reaction can be coupled to the reduction of quinones. The PMS photoreactions catalyzed by both chloroplasts and isolated chlorophyll respond in like manner to type of detergent present and pH of the medium, suggesting a direct participation of the chlorophyll in the chloroplast when the intact chloroplast is used. This is consistent with the unique response of PMS to light intensity in the photophosphorylation reactions listed above [31, 32] and also agrees with the observation made by the Marburg group [3] that when concentrations of reduced PMS larger than 10^{-5} M are used, the PMS bypasses the cytochrome and reacts directly with the chlorophyll a.

It is apparent that the reaction described in these experiments is complex. It is influenced by the nature of the detergent and the pH of the medium, indicating it is sensitive to a change in the charge composition of the medium. Although interesting speculations can be made concerning the correlation of detergent type (anionic, neutral and cationic detergent) and the reactions catalyzed, it would be premature to so extend the present data. It is well known that the photochemical properties of dyes are markedly changed by the environment in which they are found. This is particularly true when the dyes are adsorbed on the surface of macromolecules as shown by Oster and Bellin [33, 34] and more recently by Kostenbauder and DeLuca, who studied the photoactivity of riboflavin in the presence of sodium decylsulfate [35].

The ability of quinones to act as electron acceptors in photochemical reactions involving chlorophyll is well known. As an example of this type of reaction can be cited the recent experiments of Tollen and Green [36, 37], who investigated electron transfer reactions between excited chlorophyll a molecules and various quinones (including UQ_6) dissolved in organic solvents. These investigators showed that electron transfer to the quinone does occur, and furthermore the structure of the quinone used as electron acceptor has a

profound effect upon the electron transfer reaction. These experiments were performed in organic solvents, but it is likely that in the qualitative sense, these data can be translated to aqueous solvents with detergents present.

Contribution No. 131 from the Charles F. Kettering Research Laboratory. The abbreviations used are as follows: NADP, nicotinamide adenine dinucleotide phosphate; UQ_2 and UQ_2H_2, ubiquinone with two isoprene units and its reduced form; UQ_6 and UQ_6H_2, ubiquinone with six isoprene units and its reduced form; TMQ, trimethylbenzoquinone; PMS and $PMSH_2$, phenazine methosulfate and its reduced form.

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FLUORESCENCE, ENERGY TRANSFER, AND SH-GROUPS IN PHOTOSYNTHETIC PIGMENTS OF RED AND BLUE-GREEN ALGAE

Eiji Fujimori and Kenneth Quinlan

The phycobilins of the red and blue-green algae have been shown to have the unique property of utilizing light energy more efficiently in photosynthesis than chlorophyll a. Duysens [1] has shown that the energy absorbed by phycoerythrin in red algae is transferred mainly to fluorescent chlorophyll, with a smaller amount to the weakly or non-fluorescent chlorophyll. Spectral studies of the phycobilins show that more than one type of chromophore exist in the chromoproteins [2]. Phycoerythrins and phycocyanins exhibit various numbers of absorption maxima in the visible which are due to different types of chromophore. At present time, the energy interactions of the chromophores within the same pigment-protein complexes as well as those between the different types of chromophore in the phycobilins and the different forms of chlorophyll are not clear. A spectral study of the phycobilins in vivo and in vitro may shed light on the function of the different chromophores in photosynthesis and their relationship to the chlorophylls.

Modifying the structure of the protein associated with the photosynthetic pigments may induce changes in the spectral and photochemical properties of the chromophores. These changes may elucidate the types of energy interaction between the different pigments. This paper reports the results of a study of the effect of the sulfhydryl blocking agent, p-chloromercuribenzoate (PCMB), on purified phycoerythrin and phycocyanin. These results are correlated with the changes induced by PCMB on the ruptured and whole cells of red and blue-green algae. Newton [3] has shown that disulfide and sulfhydryl groups play a role in the energetics of photosynthetic bacteria.

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PHYCOERYTHRIN

Treatment of purified phycoerythrin from Ceramium rubrum with PCMB eliminates the 565 mμ absorption band from the visible spectrum [4]. This same effect has been found in the present study with purified phycoerythrin from Porphyridium cruentum. Fig. 1 shows the disappearance of the 565 mμ band with the 500 mμ and 545 mμ absorption bands remaining intact. The latter two absorption bands disappear on reduction with sodium hydrosulfite. Evidence obtained from the present study shows that a segment of the phycoerythrin containing the 500 mμ chromophore can be separated from a segment containing the 545 mμ and 565 mμ chromophores. This result will be described below. These studies illustrate the existence of three types of chromophore in phycoerythrin and the influence of sulfhydryl groups on the 565 mμ chromophore.

The addition of PCMB to native phycoerythrin decreases the fluorescence intensity with time. This result indicated that the 565 mμ chromophore is responsible for the radiative transition since the absorption maximum at 565 mμ is the only one effected by PCMB. The action spectrum of the fluorescence (Fig. 2) shows also the participation of the 500 mμ and 545 mμ chromophores in transferring energy to the 565 mμ chromophore. A series of action spectra of the fluorescence of phycoerythrin in the presence of PCMB presented in Fig. 2 shows the decrease of energy transfer among the chromophores. Since the 500 mμ and 545 mμ chromophores can still be excited in the presence of PCMB (Fig. 1), the absorbed energies must now be dissipated in a non-radiative transition.

The sulfhydryl content of phycoerythrin has been determined by amperometric titrations (Fig. 3). A value of eight sulfhydryl groups per molecule of phycoerythrin has been obtained, assuming a molecular weight of 290,000 [5] and a specific extinction coefficient of 2.73 at 565 mμ [6] for phycoerythrin. It is not known what fraction of these sulfhydryl groups are associated with the chromophore.

PHYCOCYANIN

The purified phycocyanin from the blue-green alga, Anacystis nidulans, exhibits an apparent single absorption peak at 615 mμ and a fluorescence at 640 mμ. However, the action spectrum of the

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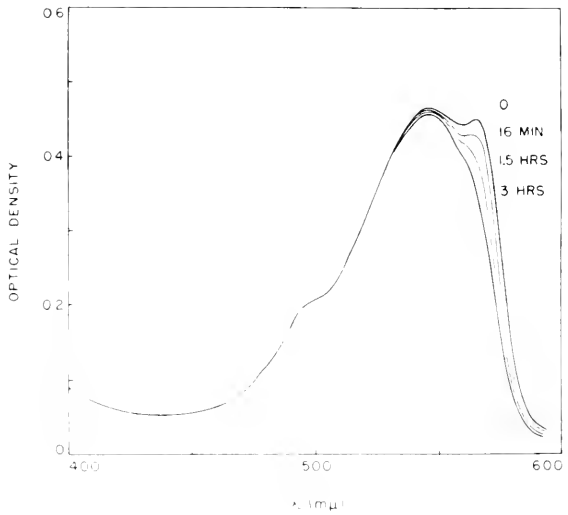

Fig. 1 Absorption spectra of phycoerythrin
(pH 7.0, 1.36×10^{-4}M PCMB)

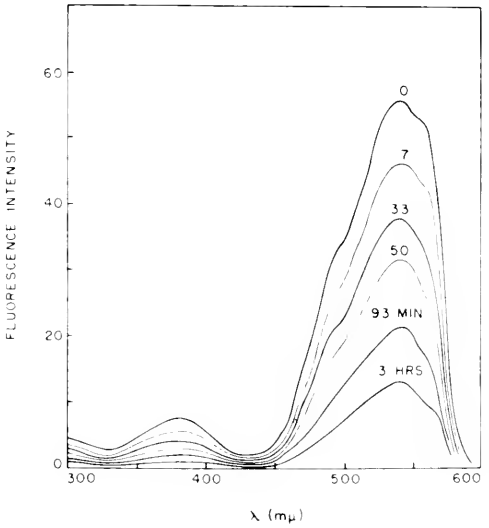

Fig. 2 Action spectra of phycoerythrin fluorescence at 570 mμ
(pH 7.0, 1.36×10^{-4}M PCMB)

fluorescence shows two distinct bands at about 590 and 620 mμ (Fig. 4) which correspond to at least two different types of chromophores. These results are in agreement with the observation of Berns, et al. [7] on phycocyanin obtained from the blue-green alga, Plectonema calothricoides. The existence of two chromophores has further been verified by the reduction of the 590 mμ chromophore with sodium hydrosulfite. The shift of the fluorescence band towards longer wave-length when excited by light longer than 615 mμ shows that both chromophores are capable of fluorescing. Their fluorescence peaks have been estimated to be at 630 and 650 mμ.

In the presence of PCMB the absorption and fluorescence of phycocyanin decreases. This decrease can be partially recovered by the addition of glutathione as shown in the action spectra in Fig. 4. The same effect has been found in phycoerythrin. The change of the spectral properties of phycocyanin on the addition of PCMB is less than that of phycoerythrin. This is in good agreement with the result showing a much smaller sulfhydryl content for the phycocyanin. Amperometric titrations show a sulfhydryl content of one per molecule of phycocyanin. The molecular weight and specific extinction coefficient used were 273,000 [5] and 7.9 at 615 mμ [6], respectively. The action spectrum of the shorter wave-length fluorescence suggests that PCMB does not influence this radiative transition (Fig. 4). The sulfhydryl blocking agent influences only the longest wave-length chromophore as found with phycoerythrin. These chromophores may in some way be bonded to the protein through the sulfhydryl group.

RED AND BLUE-GREEN ALGAE

The present study has shown that the longest wave-length chromophore of the purified phycobilins is associated with sulfhydryl groups since PCMB inactivates the longest wave-length absorption. This particular chromophore is also capable of receiving energy from the shorter wave-length chromophores. This investigation was extended to observe the effect of PCMB on the spectral behavior of phycobilins and chlorophylls in the whole and ruptured cells of red and blue-green algae. These results may give additional information on the role of accessory pigments and

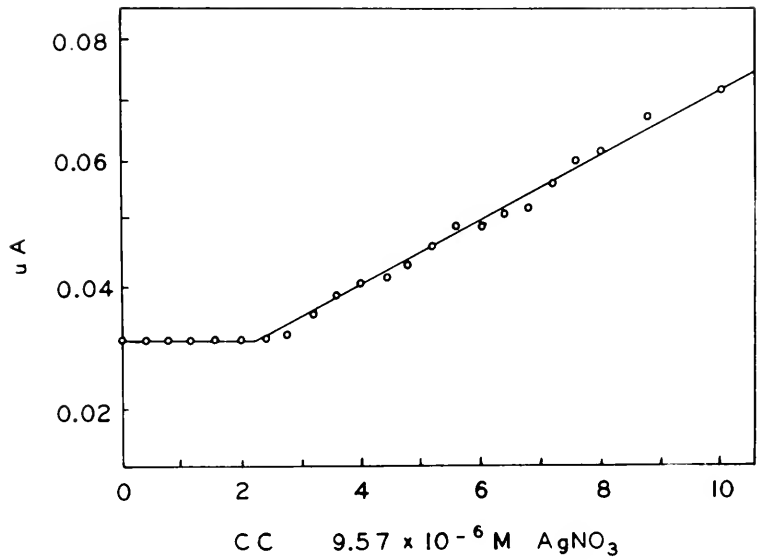

Fig. 3 Amperometric titration curve of phycoerythrin (pH 7.0, 1.095×10^{-1} g/l, 7 cc.)

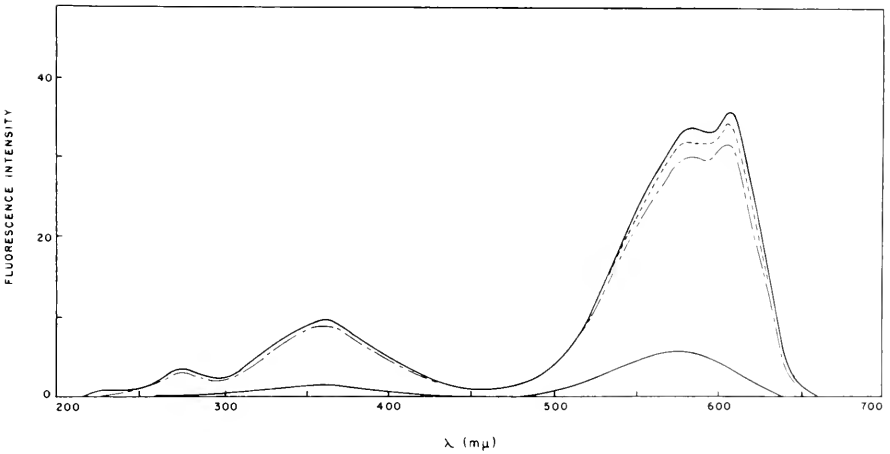

Fig. 4 Action spectra of phycocyanin fluorescence at 645 mμ (upper solid curve) and at 610 mμ (lower solid curve). - — - — -, 6.8×10^{-5}M PCBM; -----, 6.8×10^{-5}M PCBM + 2×10^{-3}M glutathione (pH7.0)

chlorophylls in the light reactions of photosynthesis.

When the cells of Porphyridium cruentum and Anacystis nidulans are ruptured by sonic oscillation and treated with PCMB, the change of absorption occurs only at the phycobilin band. The absorption and fluorescence spectra of the phycobilins undergo the same changes as the purified phycobilins. The fluorescence of chlorophyll excited by the phycobilins also decreases. This decrease in fluorescence of chlorophyll can be explained by the decrease in efficiency of the energy transfer from the phycobilins to chlorophyll, since the longest wave-length chromophore becomes inactive.

The effects of PCMB on intact cells of both algae are different. Fig. 5 shows the gradual increase of fluorescence of phycoerythrin in Porphyridium cruentum and the initial increase of chlorophyll fluorescence followed by a subsequent decrease when phycoerythrin was excited. Fig. 6 exhibits the continuous decrease of phycocyanin fluorescence and the increase of chlorophyll fluorescence in Anacystis nidulans. The increase in fluorescence of the phycoerythrin in Porphyridium cruentum has been found to be due to the release of a modified fluorescent phycoerythrin from the cell by PCMB. This modified fluorescent phycoerythrin is different from the non-fluorescent one formed in isolated phycoerythrin treated with PCMB. Anacystis nidulans does not release any pigment. The modified phycoerythrin exhibits only the 545 mμ band with the inactive 565 mμ band. The 500 mμ chromophore component was found to remain in the cell. The studies of modified fluorescent phycoerythrin will be reported elsewhere. The separation of modified phycoerythrin from the cell explains the subsequent decrease of energy transfer from phycoerythrin to chlorophyll. The initial increase of the fluorescence of chlorophyll in Porphyridium cruentum and the continuous increase in Anacystis nidulans may be the result of an efficient direct energy transfer from the shorter wave-length chromophores to chlorophyll. It is also suspected that the structural environment or arrangement of chlorophyll may have been changed, since a continuous increase in chlorophyll fluorescence is observed in the PCMB-treated whole cells when chlorophyll is directly excited with blue light.

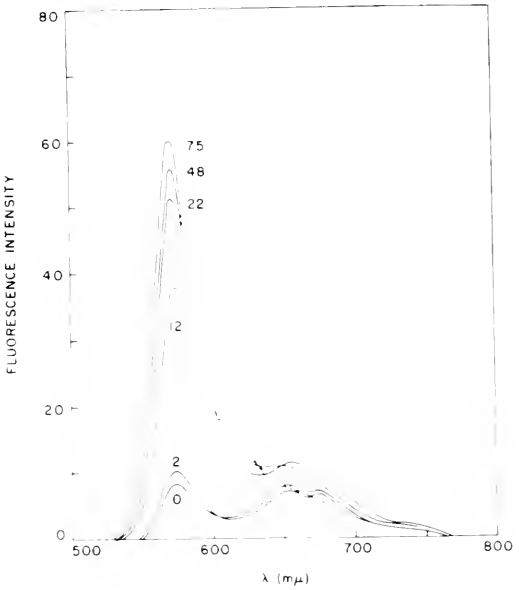

Fig. 5 Fluorescence spectra of Porphyridium cruentum. (pH 7.0, 6.8×10^{-5}M PCMB, Excitation at 500 mμ)

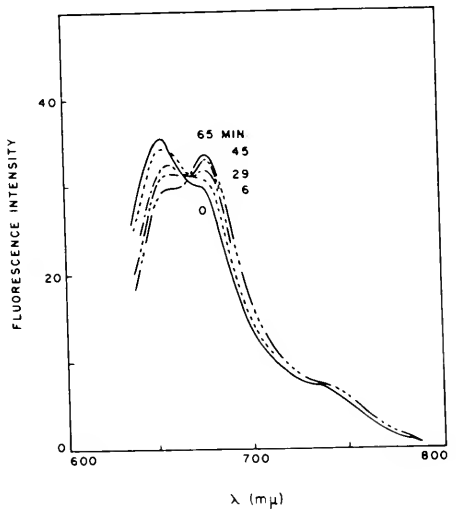

Fig. 6 Fluorescence spectra of Anacystis nidulans. (pH 7.0, 6.8×10^{-5}M PCMB, Excitation at 615 mμ)

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In summary, additional evidence is presented for the presence of different types of chromophore in the phycobilins. The longest wave-length chromophore of the phycobilins interacts with sulfhydryl groups of protein. It was shown that the longest wave-length chromophore is capable of receiving energy from the shorter wave-length chromophores, acting as a bridge in transferring the energy to chlorophyll. When the longest wave-length chromophore is inactivated by PCMB, the energy transfer from the shorter wave-length chromophores to chlorophyll is interrupted, as shown in ruptured cells. The studies of whole cells suggest the existence of the direct energy transfer from the shorter wave-length chromophore to chlorophyll in vivo.

ACKNOWLEDGMENT

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STUDIES OF THE LOCALIZATION, PHYSICOCHEMICAL PROPERTIES, AND ACTION OF PHYCOCYANIN IN ANACYSTIS NIDULANS

John A. Bergeron

The blue-green algae, classed as primitive monerans[1], represent the lowest level of organization known to possess a higher plant type of photosynthesis. The role in photosynthesis of the phycocyanin in these organisms is particularly interesting from the point of view of ultrastructural biochemistry. It has been shown repeatedly[2-7] that despite the primacy normally assigned to chlorophyll, the light absorbed by phycocyanin is used more efficiently for photosynthesis (equated with photosynthetic oxygen evolution). This property is all the more intriguing since Hill activity is very labile in these organisms; the loss being correlated with the release of phycocyanin[8]. Then too, it has been observed that fluorescence at about 685 mμ, attributed to chlorophyll, is proportionately greater for wavelengths absorbed by phycocyanin. French and Young[9] attributed the differential to inactive absorption by carotenoids in the "Soret" region of chlorophyll, but Duysens[3] concluded that the magnitude of the difference observed by him was too great to be explained by screening. Duysens proposed the existence of two pools of chlorophyll of about equal size; one containing fluorescent, photosynthetically active chlorophyll in proximity to phycocyanin, the other nonfluorescent, photosynthetically inactive and remote from phycocyanin.

We have studied the blue-green alga Anacystis nidulans in the hope of obtaining a better understanding of these phenomena. This unicellular organism is characterized by a high capacity for photosynthetic oxygen evolution, minimal dark respiration and a generation time, under favorable conditions, of about two hours [10,11]. The organism is maintained in continuous culture in the D medium[11] at 25° or 35° C in cylindrical growth chambers of one cm light path thermostated by circulating water through inner and outer jackets. The light source is a 40 W daylight fluorescent tube located on the axis. The culture is kept turbulent by the introduction of the gas mixture (1-5% CO_2 in air) via a medium porosity frit located at the bottom to one side.

John A. Bergeron

LOCALIZATION AND INTRACELLULAR CONCENTRATION OF THE PHOTOSYNTHETIC PIGMENTS

It is important to know not only the intracellular localization of phycocyanin, but also the relationship of phycocyanin to the chlorophyll-bearing structures. Electron microscopic observations on a number of blue-green algae[12-21] have established the presence of triple-layered cytoplasmic lamellae as a consistent feature. It is known that the chlorophyll containing particles, free of phycocyanin, obtained by fractionation of broken cells consist of lamellar fragments[13]. While it is clear that the cytoplasmic membranes contain chlorophyll and that the integrity of these membranes does not depend upon a fixed relationship with phycocyanin; the relationship of these membranes to phycocyanin and the physical basis for the concept of two pools of chlorophyll has remained obscure.

Our approach to these questions has been based upon the following types of observations; spectrophotometric studies of the pigments coupled with cell counts and cell volume determinations, dark field fluorescence microscopy of normal organisms and organisms repeatedly extracted in 80% acetone, and electron microscopy of intact organisms and subcellular preparations.

The concentration of chlorophyll *a* and phycocyanin in cell suspensions was calculated by the procedure of Kratz and Meyers[10] from the absorption spectra. Opal glass was used to reduce the effect of light scattering. The concentration of organisms in the suspensions was calculated from measurements with a Petroff-Hausser bacteria counter. These numbers suffice to calculate the pigment content of the cells. For typical type E cells[10] from log phase the average chlorophyll content was 2.05×10^{-11} mg/cell or 1.3×10^{7} molecules. The phycocyanin content was 1.52×10^{-10} mg/cell or 3.27 to 6.54×10^{5} molecules depending upon the assigned molecular weight (see below). An average cell volume was calculated from measurements (n = 50) of the length and diameter of these cells from phase contrast micrographs taken at known magnification and enlarged upon Resisto paper. From the mean diameter (0.92 ± 0.01 μ) and length (2.67 ± 0.82 μ) the mean volume was calculated as the sum of two hemispheres and a right cylinder (H = L - D). The average volume was 1.58×10^{-12} cc. On this volume basis the intracellular pigment concentration, expressed over the entire cell, is 1.44×10^{-2} *M* for chlorophyll and 3.44 to 6.88×10^{-4} *M* for phycocyanin.

It is easy to observe, but difficult to record by photography,

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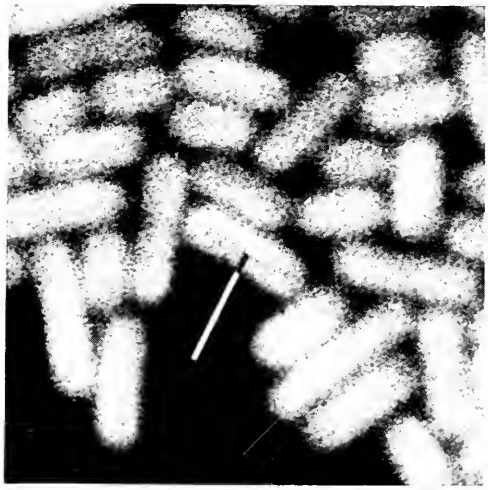

Fig. 1. Dark field fluorescence micrograph of extracted Anacystis showing nonfluorescent axial region.

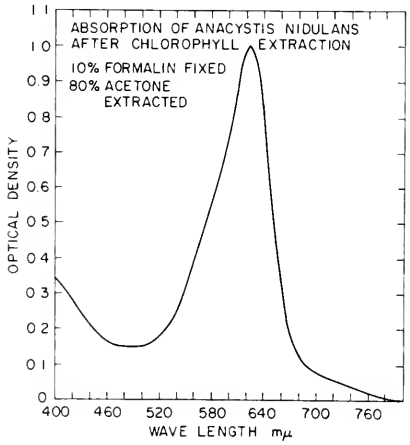

Fig. 2. Absorption spectrum of Anacystis repeatedly extracted in 80% acetone after 15 min exposure to 10% formalin.

that the red fluorescence of phycocyanin and chlorophyll is restricted to the cortex of the organism. This is still true when enough chlorophyll has been extracted so that the pigment is no longer observable in the absorption spectrum (Figs. 1 & 2). It seems appropriate to conclude that the bulk, if not all, of the fluorescent chlorophyll and phycocyanin is in the cortex.

This result is not surprising since the axial region of Anacystis resembles the bacterial nucleoid. In our cultures the fine structure of organisms taken from the log phase of growth is typified by the example in Figure 3. The peripheral cytoplasm is incompletely partitioned by two concentric membranes spaced about 500 Å apart. There is also present a third incomplete membrane. Employing Figure 3 as an example of an axial section we have attempted to assess whether the cytoplasmic membranes could account for all of the chlorophyll and also the effect of limiting the phycocyanin to the cortex. From the volume (8.0×10^{-13} cc) calculated for this cell compared with the concentrations calculated for the average cell (volume of 1.58×10^{-12} cc) this cell would

contain 6.94×10^6 molecules of chlorophyll. The lamellar area available for chlorophyll (each membrane has two sides, the innermost is treated as a half) is 1.83×10^9 Å^2 or 272 Å^2 per molecule. Since the area of the "head" of the chlorophyll molecule is about 225 Å^2 all of the chlorophyll could be accommodated in the cytoplasmic membranes which occur in the fluorescent cortical region.

The cortical volume was calculated (6.43×10^{-13} cc) and used to estimate the cortical concentration of phycocyanin (4.24 to 8.52×10^{-4} M). At this concentration the maximum possible uniform separation between adjacent molecules is between 124 and 156 Å. For spherical protein molecules in the molecular weight range of 1.4 to 2.8×10^5 gm/mole the diameter would be 70 to 90 Å. This maximum possible uniform spacing would leave the molecules less than a molecular diameter apart. It seems likely that the phycocyanin molecules exist between the lamellae in a state in which molecular interaction is probable. These observations are not consistent with the hypothesis that phycocyanin is related to one pool of chlorophyll and remote from another of equal size. Another explanation should be provided to account for the apparently greater efficiency of phycocyanin in producing chlorophyll fluorescence (see below).

PHYSICOCHEMICAL STATE OF PHYCOCYANIN IN VIVO

It is known[22-24] that phycocyanin dissociates reversibly in the pH range between 6 and 7. Apparently investigators interested in the photosynthetic activity of this protein have not considered the potential significance of this property.

Phycocyanin was released from Anacystis nidulans by rupturing the organisms in a French pressure cell in phosphate buffer (0.1 M, pH 5.7). The purification, excepting the precaution of not allowing the pH to rise above 5.7, followed the procedure of Hattori and Fujita[24]. Figure 4 illustrates that there is a decrease in absorption of the 621 mμ maximum and a broadening of the band width over the pH range where dissociation occurs. The fluorescence of the protein also changes. Figure 5 illustrates that the dimer possesses a fluorescence maximum at 680 mμ which is depressed for the monomer. When molecular interaction is enhanced by the addition of ammonium sulfate, the 660 mμ maximum is depressed leaving the 680 mμ band as the maximum. By contrast, monomeric phycocyanin, similarly treated, does not show a comparable alteration of the fluorescence spectrum. These observations provide an alternative explanation for the results obtained for the fluorescence of blue-green algae since purified

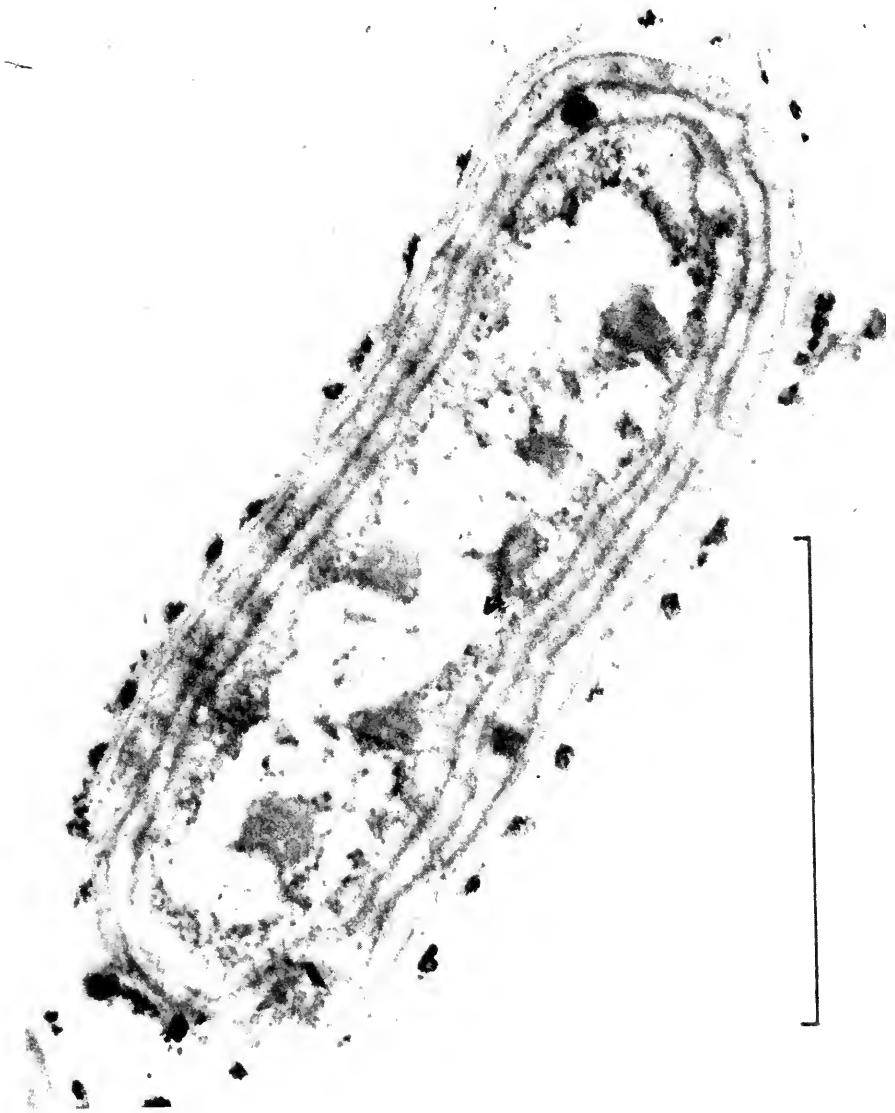

Fig. 3. An electron micrograph of an axial section of Anacystis nidulans. The cortex is incompletely partitioned by three concentric membranes spaced about 500 Å apart (scale = 1 μ).

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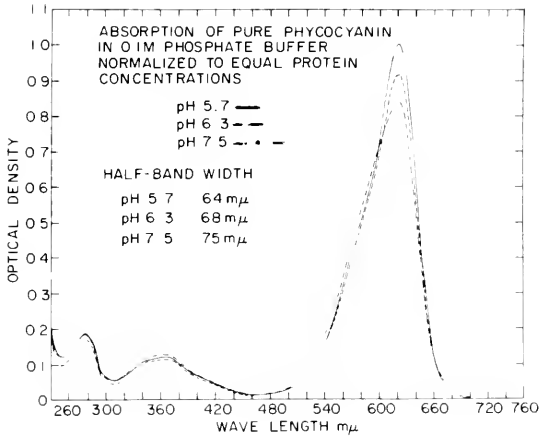

Fig. 4. Effect of pH on the absorption of purified phycocyanin.

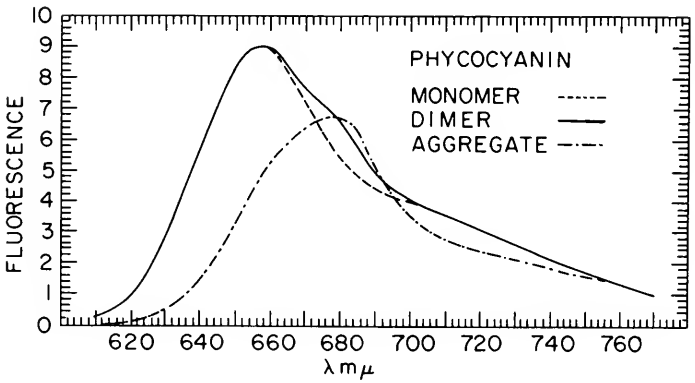

Fig. 5. Fluorescence of pure phycocyanin in several states excited at 578 mμ.

phycocyanin can exhibit a fluorescence maximum very close to the fluorescence of chlorophyll *in vivo*.

When *Anacystis* is extracted repeatedly with 80% acetone and returned to buffer, the fluorescence excited at 578 mμ or 436 mμ

has a well defined 680 mμ component (Fig. 6). If this were due to chlorophyll it should be exaggerated with 436 mμ excitation

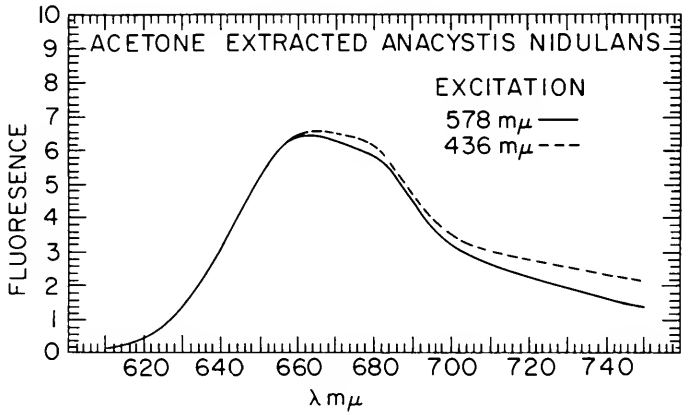

Fig. 6. The fluorescence of acetone extracted Anacystis.

and depressed with 578 mμ excitation. Since the two curves are almost identical we conclude that most of the 680 mμ emission of the extracted organisms is not due to chlorophyll and that, therefore, the phycocyanin is in a state which resembles the effect of aggregation on a mixture of monomeric and dimeric phycocyanin. If the fluorescence of phycocyanin in vivo is similar to the emission observed in this case for the residue then the enhanced fluorescence at 680 mμ is explained. Figure 7 illustrates the fluorescence of Anacystis at room temperature for equal quanta absorbed at 436 mμ and 578 mμ for a very dilute suspension. We have superimposed the fluorescence of the extracted organisms, monomeric phycocyanin and dimeric phycocyanin. Depending upon the curve selected to represent the contribution of phycocyanin to the fluorescence excited by 578 mμ light, it could be concluded that energy absorbed by phycocyanin is between 1 to 4 times as effective in exciting chlorophyll as light absorbed at 436 mμ. If we consider screening by carotenoids then light absorbed by phycocyanin probably is 50 to 100% as effective in exciting chlorophyll fluorescence as light absorbed directly by chlorophyll.

ACTION OF PHYCOCYANIN IN PHOTOSYNTHESIS

The essence of the preceding paragraphs is that the enhanced activity of phycocyanin in photosynthetic oxygen evolution can

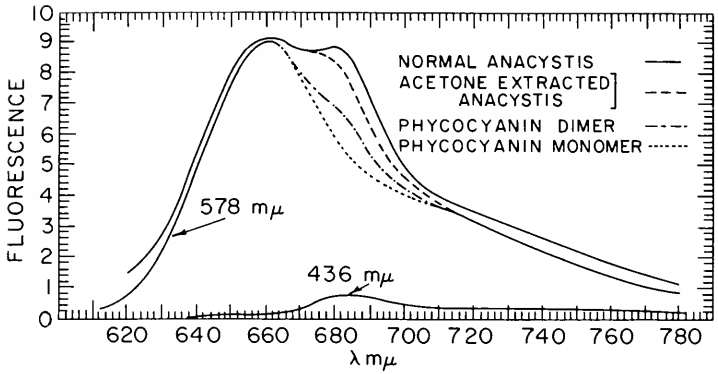

Fig. 7. Fluorescence of Anacystis nidulans per quantum absorbed at 578 mμ and 436 mμ compared with the fluorescence of extracted organisms and pure phycocyanin in the monomer and dimer states.

not be attributed to action by way of the bulk of the chlorophyll.

We have used fluorescence studies at low temperature[25] (liquid nitrogen) in an attempt to determine whether light absorbed by phycocyanin is preferentially transferred to a species which is not well excited by way of the chlorophyll--as Figure 8 shows, such a species exists. In Anacystis there are in addition to phycocyanin and chlorophyll two species which are very weakly fluorescent at room temperature but appear as major emission bands at liquid nitrogen temperature; one at 700 mμ, the other at 720 mμ. On the basis of location, preferential excitation via chlorophyll, and ferricyanide bleaching, we have tentatively equated the latter species with the P-700 of Kok and Hoch[26], the oriented 705 pigment of Olson and Butler[27,28], and the oriented 695 chlorophyll of Sauer and Calvin[29]; that is, the energy acceptor or sink for system one of photosynthesis. By analogy, we infer that the 700 mμ fluorescence which is preferentially excited by phycocyanin may be the energy sink for the oxygen evolving system or system two. If this species absorbs on the short wavelength side of the ordinary chlorophyll a like so-called chlorophyll-670[30] then poor overlap with the bulk of the chlorophyll would account for poor excitation by chlorophyll but excellent excitation via phycocyanin.

Within the framework of the concept of two spectrally different

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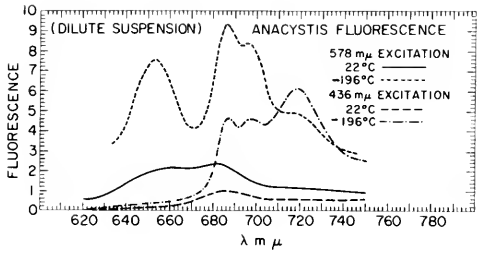

Fig. 8. Effect of temperature on the fluorescence of Anacystis. Two new bands appear at -196° C, one at 700 mμ, the other at 720 mμ.

energy sinks which straddle the red absorption maximum of the bulk of the chlorophyll a it is possible to explain the energy flow between the two light reactions of higher plant photosynthesis by means of sensitized fluorescence. Presumably the phycocyanin and phycoerythrin of blue-green and red algae and the chlorophyll b of higher plants fill the role of funneling energy into the energy acceptor of system two.

ACKNOWLEDGEMENTS

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VII. CHLOROPLAST STRUCTURE AND ORIENTED MOLECULES

EXPERIMENTS MADE TO ELUCIDATE THE MOLECULAR STRUCTURE OF CHLOROPLASTS

Wilhelm Menke

It has been known at least since 1940 that the chloroplasts possess a lamellar fine structure[1, 2, 3, 4]. It has also been known since that time that the grana are not independent structural elements, but differentiations of the lamellar system[5]. Not until 20 years later was the general morphological structural principle of the lamellar system discovered[6, 7, 8, 9]. Fig. 1 shows the most important types of these lamellar systems in bacteria, cyanophyceae, algae and higher plants in schematic representation. The structural

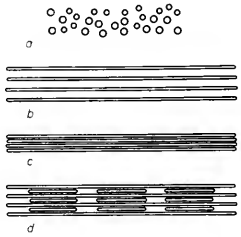

Fig. 1. Main types of the photosynthetic lamellar system.
a- Purple bacteria; b- Cyanophyceae and Rhodophyceae;
c- Grana-free chloroplasts; d- Grana-containing chloroplasts

elements of the lamellar system are in every case membranes closed sack-like in themselves, for which I have suggested the name "thylakoids". Thylakoids are usually flattened, but may sometimes be swollen like vesicles. We know that the photochemical reactions of photosynthesis and electron transport take place within the thylakoid membranes[10].

A certain uniformity in chemical structure appears to correspond to the uniform morphological structure principle. Thylakoids consist of proteins, lipids and a number of other compounds. After the extraction of the lipids (about 45-48% of the dry weight) and washing of the residue with water and saline solutions about 52-55% of the dry weight of the thylakoids remains. This contains about 80-85% amino acid residues, 8-10% carbohydrates and 1% nucleotides. Part of the carbohydrates - among which arabinose and galactose predominate - can be split off by treatment with phenol. The amino acid composition of the lamellar structural protein thus obtained is remarkably

alike in the case of all plants examined so far (Fig. 2)[11, 12]. Terminal group determinations showed that a considerable number of different polypeptide

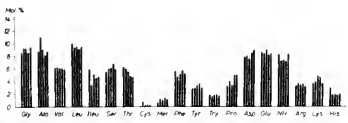

Fig. 2. The amino acid composition of the lamellar structural proteins from thylakoids of different plants (left to right for each amino acid) Oscillatoria chalybea, Chlorella pyrenoidosa, Allium porrum, Spinacia oleracea, Antirrhinum majus

chains is involved in the build-up to the lamellar structural protein[13]. It has so far been possible to dissolve the lamellar structural protein only in anhydrous formic acid. It is decomposed into subunits in this process, which sediment fairly uniformly in the ultracentrifuge at 5.9 S. The particle weight is about 165, 000[14]. Further decomposition takes place during oxidation with performic acid[14].

The composition of the lipid mixture is not so uniform in various plants, although it also has common and characteristic features. I would draw attention here to the presence of galactolipids and the predominance of linolenic acid among the fatty acids[15, 16, 17, 18, 19, 20]. The thylakoids contain 8-10% chlorophyll. The quantitative chemical analysis of chloroplasts and their lamellar system is but one of the ways to be adopted in elucidating the molecular structure.

It is known that chloroplasts are double refracting and dichroic. The fluorescence emitted by chloroplasts is in part linearly polarized[21, 22]. The analysis of optical anisotropy showed already quite some time ago that the chloroplasts possess lamellar fine structure, and that the lamellar system probably consists of protein and lipid layers which alternate with each other, the lipid molecules being oriented perpendicularly to the planes of the lamellae[1]. The same result can be derived from volumetric studies (Fig. 3). If

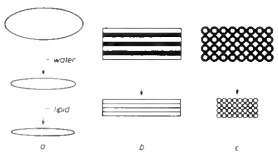

Fig. 3. a- Changes in size and shape of chloroplasts of Plagiochilla asplenioides due to loss of water and lipid extraction; b- The layer model; c- The particle model

chloroplasts, the water of which has been extracted by preservative drying, are extracted with organic solvents, their diameter remains unchanged, while their thickness is reduced to almost half. This must be the case if proteins and lipids are present in separate layers. The diameter of mixed layers of

lipids and proteins would have to decrease upon extraction of the lipids. The water content of chloroplasts was also determined by volumetric studies. Chloroplasts contain 65-80% of water, about three-quarters of which is embedded interlamellarly[23].

We have been engaged for a number of years - initially in collaboration with Professor Kratky in Graz[24], and later on in Cologne with Dr. Kreutz - in studies aimed at obtaining further information about the structure of the lamellar system by X-ray small angle scattering. We hoped thus to be able to determine the thickness of the individual layers and the period of thylakoid stacking, and perhaps also to discover something about the structure of the layers.

We began by determining the thickness of the protein layers from the diffuse X-ray small angle scattering[25]. Evaluation of the scattering curves is comparatively simple only if the protein layers lie individually distributed in the preparation without any preferred orientation. In order to obtain preparations which largely satisfy these requirements, the chloroplasts were washed thoroughly with water, freeze-dried, extracted with organic solvents, washed again with water, freed from rapidly sedimenting portions, and freeze-dried once more. The analysis of scattering curves, which was effected by various procedures, showed that a lamellar system was present in the preparations, and that the protein layers are 48 ± 2 Å in thickness. It was, however, discovered that the preparations in general contain not only individual layers but also stacks consisting of a small number of protein layers. It was discovered later on that the scattering curves display a broad maximum, corresponding to a Bragg's value of about 36 Å. The only explanation of this maximum is that there is a periodicity within the plane of the protein lamellae. This periodicity manifested itself repeatedly also later on, when we had changed over to examining chloroplasts in an aqueous suspension and in living cells. The best coincidence between the computed and experimental scattering curves was found upon assumption of a quadratic lattice consisting of particles scattering equivalent to spheres of 31.4 Å in diameter. The lattice arrangement, however, does not extend over the whole protein layer but is restricted to crystallites. Each crystallite probably consists of 25-30 particles. The crystallites are joined loosely or else separated from each other by amorphous intermediate regions[28].

It appeared natural to determine next the thickness of the non-extracted thylakoid membranes in freeze-dried preparations. Analysis of the scattering curves showed a value of 48 Å in this case as well. This surprising result is explained by disorganization of the lipids, which takes place during drying. Films of chloroplasts dried onto glass also showed some interferences attributable to secondary crystallization of some components of the disorganized lipid mixture[26]. Lipid crystals are visible in the polarization microscope under intensive illumination.

Since loss of water obviously results in partial destruction of the structure of thylakoid membranes, isolated chloroplasts in an aqueous suspension were examined next[27]. These produced usable scattering curves which, however, varied to a certain degree between one preparation and another. In addition scattering curves of living Chlorella cells were recorded, which in turn differed from the scattering curves of isolated chloroplasts (Fig. 4). It was now a case of finding models for the individual scattering curves, the computed scattering of which would coincide with the experimental scattering curves. These models should be of such a nature, that they all derive from one basic

model by slight, plausible alternations. This basic model, on which the scattering curves are based, has the following properties: it consists of mem-

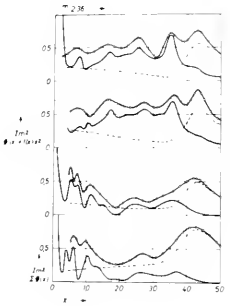

Fig. 4. Small angle scattering curves of isolated chloroplasts (Antirrhinum majus), above, and living cells (Chlorella pyrenoidosa), below.

—o—o— experimental curve
——— calculated curve
-------- difference between experimental and calculated (mean values)

branes, each of which is composed of two layers of different electron density. These asymmetric membranes are oriented pairwise enantiomerically toward each other. One layer is continuous, the other of corpuscular structure. According to the former results the corpuscular layer is protein, and the continuous layer is lipid material. The dimensions of the model and the period of stacking can be gathered from Fig. 5. The differences between the

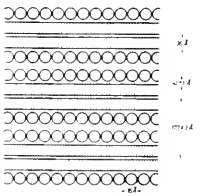

Fig. 5. Schematic transversal section through a thylakoid stack

individual experimental scattering curves are explained by a certain variability in the period and by statistical fluctuations in distance. A further point to be taken into consideration in isolated chloroplasts of Antirrhinum is the scattering of the regions of the lamellar system situated between the grana, and in Chlorella the scattering of the matrix and of the remaining cell structures. The constant component is in every case the scattering of the pairs of

polarly-built thylakoid membranes which are oriented enantiomerically to each other.

The next point to be clarified was the manner in which the individual thylakoid membranes are linked to the thylakoid, i. e. whether the protein layer or the lipid layer forms the external boundary of the thylakoid[29]. This question could be answered since it proved possible to obtain films of oriented chloroplasts in which no noticeable disorganization of lipids had taken place, by drying a chloroplast suspension onto glass plates. In such films the thylakoids are situated approximately parallel to the film-plane. From the point of view of polarization optics the whole film behaves like an uniaxial double-refracting crystal plate. The optical axis emerges vertically to the film-plane. When the support is removed, such films will, if they contain a certain amount of water, roll up into little tubes. These tubes were found serviceable for further investigations. They produced a series of interferences (Plates 1, 2) the first order of which, depending on the degree of drying, corresponds to Bragg values of 152-166 Å. The interference of the first order is very weak, the second order the strongest. This series of interferences is caused by a period parallel to the film normal. Moreover, all the preparations display a faint, diffuse ring at 36.5 Å. This is intensified at the equator and is therefore produced by a periodicity in the plane of the thylakoid membrane.

Fig. 6 shows scattering curves of a preparation photographed with a small angle camera according to Kratky (Fig. 7). The little tubes are oriented

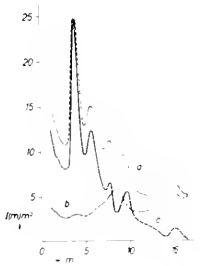

Fig. 6. Scattering curves of layers of oriented chloroplasts dried on glass plates.

a Scattering in the meridian
b Scattering in the equator
c Scattering caused by the stacking of thylakoids

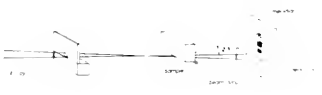

Fig. 7. Kratky-camera with quadratic cross section of the X-ray

horizontally. The X-ray had a quadratic cross-section. Curve 6a reproduces the scattering in the meridian, while curve b corresponds to the equatorial scattering. (The preparation was oriented vertically for photograph b.) The curve was multiplied by m^2 ($m \sim \vartheta$; I = intensity) in order to eliminate the leaflet factor. Curve c was obtained by subtraction of curve b from curve a. It indicates the scattering caused by the stacking of thylakoids.

The scattering curve 6c is to be decomposed into a form factor and a lattice factor. The form factor corresponds to the scattering of the individual thylakoid and is therefore calledthylakoid function here. The lattice factor takes into account the stacking of the thylakoids. In the present case the task is that of finding the thylakoid function. Since the lattice factor has a constant value in the maxima of the scattering curve, the intensities of the scattering curve at these maxima is determined by the thylakoid function, therefore the maxima of the scattering curve are points of the thylakoid function. Fig. 8a shows the model, the scattering curve of which most closely approximates the thylakoid function. Fig. 8b shows the amplitude function of this

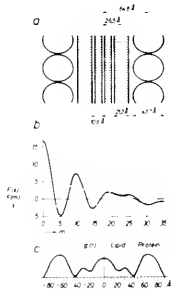

Fig. 8. b o—o Theoretical amplitude function of the model a.
o···o Values taken from the experimental scattering curve.
c Distribution of electron density parallel to the surface normal of the thylakoid (Fourier-analysis) φ (r) in relative units.

model. The values taken from the experimental scattering curve have been drawn in for comparison, choosing the signs according to the model. On the basis of this approximate amplitude function it was possible to compute the distribution of electron density (φ(r) parallel to the surface normal of the thylakoid. The unidimensional Fourier-analysis gives the distribution of electron density shown in Fig. 8c. Quantitative estimation of the electron densities of the components participating in the building up of the thylakoids leads to the result that the two outer zones of maximum electron density correspond to the protein layers, which therefore form the external boundary of the thylakoid. Their thickness amounts to 47 Å each and thus agrees with the value obtained formerly. This conformity is all the more remarkable because the thickness of the protein layer had previously been computed under the assumption of a continuous layer. A thickness of 42 Å each results for the lipid films. The whole thylakoid is 178 Å in thickness.

If we take into consideration all of the available data we come to the model reproduced in Fig. 9. This model does not reproduce all the details of the

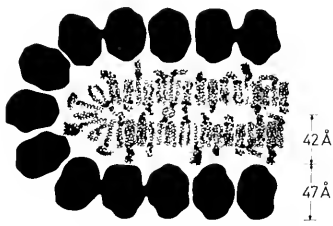

Fig. 9. Model of the thylakoid.

electron density distribution in the lipid layer. It must, however, be noted that the preparation used here has also undergone secondary changes to a certain extent, and that the scattering curve analysis was in no case possible without hypotheses. The model has chlorophyll in two different types of association that could correspond to the 670 and 683 mu forms of chlorophyll a in vivo. Only one form is in the neighborhood of protein. In both layers it is possible to have individual chlorophyll molecules as well as their polymers.

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-36Å

Plates 1 and 2. X-ray diagrams of oriented air-dried Antirrhinum chloroplasts. Kiessig camera. The little tubes are oriented horizontally. Distance sample - 20 and 40 cm.

ORIENTED MOLECULES AND THE STRUCTURE OF CHLOROPLASTS

R. A. Olson
with the collaborative assistance of W. H. Jennings

The molecular organization of chlorophyll within the chloroplast is suggested by microscopy of chloroplast structures and by the physical-chemical properties of chlorophyll preparations. The orderly fine structure of chloroplasts revealed by electron microscopy is one of the most regular of any organelle. The anatomical implication of molecular organization of chlorophyll within the chloroplast however, arises mainly from the presence of the pigment at the site of an orderly, repetitive array common to systems, i.e., crystals and mesophases, where such organization is known to exist.

Investigations of certain physical and chemical properties of chlorophyll preparations have demonstrated the capacity of the molecule to form dimers at sufficiently high concentrations and to organize monomolecular films at air-water interfaces.[1,2] Adsorption of chlorophyll on mesophase preparations yields pronounced anisotropic optical properties.[3,4] Within the practical limits of resolution, the several techniques for detecting optical anisotropy provide a sensitive method for studying molecular organization. Both bifluorescence (polarized emission) and dichroism (polarized absorption) have been observed in preparations of chlorophyll adsorbed on ammonium oleate or on lecithin mesophases. These observations indicate an orientation of the chlorophyll molecules which depends upon the molecular organization of the adsorbate molecules constituting the mesophase.

The study of the optical anisotropy of chlorophyll within the chloroplast has been far less rewarding than the mesophase observations. Among the earliest investigations, Menke (1938) used imbibition procedures to alter the birefringence and the weak dichroism of Closterium chloroplasts.[5] Considering the chloroplast a uniaxial negatively birefringent body (the optical axis lies perpendicular to the chloroplast face), he concluded that the textural or morphic anisotropy arose from a lamellar fine structure with repetitive planes parallel to the flat face of the organelle. This hypothesis is now confirmed by visual microscopy and has been further elaborated by electron microscopy. Continued studies by Menke and others of chlorophyll dichroism and anomalous dispersion of birefringence yielded evidence for a low intrinsic anisotropy in vivo.[4-8] Goedheer (1957), studying the ribbon shaped Mougeotia chloroplast, measured a maximum dichroic ratio of 1.13 ± 0.05 at 680 mμ.[4] He proposed that the chlorophyll molecules were complexed with a globin framework in such a way that they were very slightly oriented.

The bifluorescence of chloroplasts in Mesotaenium, Elodea, Mnium, and Oenothera was reported by Menke in 1958.[9] The red fluorescence from chloroplasts observed in profile aspect showed a partial linear polarization with the E vector parallel to the lamellae. Menke attributed this effect to a statistical,

partial orientation of the porphyrin oscillators in the plane of the lamellae. While the studies of anisotropic optical properties of in vivo chloroplasts have not ruled out the possibility of the molecular orientation of chlorophyll, they have not appeared to provide conclusive evidence thereof in any of the spectral regions investigated. The question is reopened, however, by the recent discovery of a far-red absorbing form of chlorophyll capable of accepting excitation energy from accessory pigments and from ordinary chlorophyll.[10] This chlorophyll demonstrates a maximum absorption near 705 mμ and a maximum emission near 720 mμ; this spectral region is at longer wave lengths than that considered in the investigations mentioned above. While conventional visual observations are limited by the poor spectral response of the human eye, suitable visual observations can be effected through use of the modern infrared image converter (the first observations reported from our laboratory were made with a surplus World War II "snooperscope"). In live Euglena cells a bifluorescence at wave lengths greater than 690 mμ was noted and in a later publication a dichroic ratio of 4.00 at 695 mμ was reported.[11,12] Using selected chloroplast fragments, Sauer and Calvin measured an electro-dichroism as high as 1.27 at 695-700 mμ.[13] The relation between these two observations awaits an understanding of the attachment site of the chlorophyll molecule. Current studies in our laboratory have been directed toward characterizing the in vivo oriented chlorophyll in detail.

EXPERIMENTAL

Dichroism

Euglena provides a most striking example of chloroplast dichroism in the far-red spectral region. Photomicrographs demonstrating this property present the conventional appearance of the organism in which the positions and spacial relationships of the chloroplasts are clearly indicated with respect to the cell outline. While the Euglena cell varies in shape from spheroid at rest to teardrop-like or prolate when actively swimming, the discoid chloroplasts are usually located peripherally, more or less facing the limiting cell membrane or pellicle. This arrangement of the chloroplasts in the cell permits the simultaneous observation, in the same preparation of numerous aspects of chloroplast orientation varying from the circular face view to the more linear edge view. It also permits a wide observational choice of edge viewed chloroplasts in numerous azimuthal aspects in the specimen plane.

The azimuthal dependence of dichroism in Euglena is demonstrated in Plate I A and B. The cells are photographed in unpolarized transmitted light from a monochromator at 695 mμ through a Nicol prism mounted behind the objective. Edge viewed chloroplasts show maximum absorption when the electric vector of the transmitted light lies in the plane of the lamellae and minimum absorption when the electric vector lies perpendicular to the plane of the lamellae. They show minimum absorption when the electric vector of the transmitted light lies perpendicular to the lamellar plane. This indicates that the oscillators of absorption lie in or very close to the plane of the lamellae. Chloroplasts

viewed from the facial aspect show slight or no detectable change in absorption regardless of the direction of the electric vector of transmitted light. This observation indicates no preferential orientation of oscillators within the plane of the lamellae.

No dichroism could be observed in the chloroplasts of non-intact or damaged cells. While chloroplasts extruded from the cell into conventional aqueous media lose their dichroism, chloroplasts extruded into viscous serum albumen retain this property, although gradually attenuated, for several hours.

In order to estimate the ratio of oriented chlorophyll to the unoriented pigment it is essential to obtain quantitative measurements of the above dichroic observations. In addition, a quantitative determination of the spectral dependence of dichroism should be executed in order to compare the spectral properties of oriented chlorophyll with those of other known pigments in vivo. The spectral dependence of dichroism is usually represented by the ratio at successive wave lengths of the absorbance parallel to the plane of the electric vector of the light maximally absorbed versus the absorbance perpendicular to that plane. Measurements of this type in vivo impose stringent requirements involving unchanging optical path length and a limited chloroplast area of measurement. Unchanging optical path length is assured by effecting a maximum degree of immobilization of the organism such that chloroplasts do not change in shape or position during the absorbance measurements at successive wave lengths. The chloroplast area selected for absorbance measurements must be very small since Euglena chloroplasts are somewhat curved and twisted and thereby show local irregularities of lamellar direction. It is therefore necessary to limit the measurement to an area small enough to exclude gross spurious effects from lamellae which lie in planes other than that selected for measurement. In our experiments this area was of the order of one square micron. The minimal radiant power transmitted from such a small specimen area when restricted to a narrow spectral band requires the measurement of an extremely low light intensity which results in an unfavorable photomultiplier signal to noise ratio. Attenuation of noise by filter circuitry is limited by time-constant characteristics imposed by the requirement of sufficiently rapid spectral scanning to minimize geometrical changes in chloroplasts caused by cell mobility.

The data are presented in terms of absorbance in Fig. 1. The upper dashed curve shows the enhanced absorbance in the lamellar direction at the longer wave lengths while the lower dashed curve shows the absorption of the unoriented chlorophyll. The ratio of the upper curve to the lower curve, the dichroic ratio, is shown by the solid curve through the triangles. In this particular specimen the maximum ratio of about 2.2 is observed near 705 mμ. Higher dichroic ratios, approaching 4.0, were obtained in other cells and in other preparations but the data offered in Fig. 1 are considered typical. Similarly, departures from the wave length of the maximum ratio in Fig. 1 were observed in other cells but the average of twelve chloroplasts measured was 704 ±5 mμ.

A similar plot of the dichroic spectral properties of the chloroplast of Mougeotia is shown in Fig. 2. While the effect in this specimen is not as pronounced as in Euglena the maximum of the dichroic ratio is located in the same

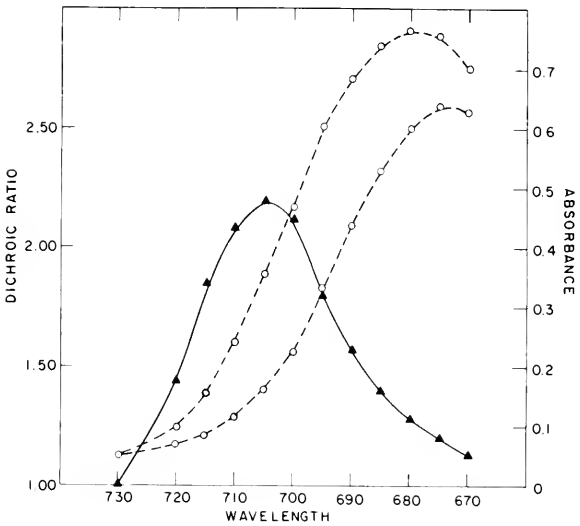
DICHROIC RATIO
2.50
2.00
1.50
1.00
730
720
710
700
690
680
670
WAVELENGTH
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE

Fig. 1

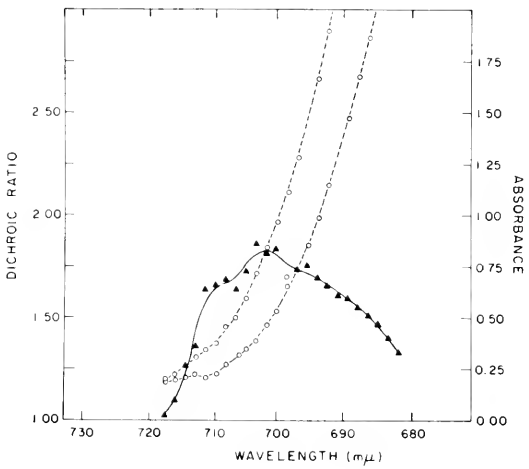
DICHROIC RATIO
2 50
2 00
1 50
1 00
730
720
710
700
690
680
WAVELENGTH (mμ)
1 75
1 50
1 25
1 00
0 75
0 50
0 25
0 00
ABSORBANCE

Fig. 2

region near 705 mμ. Similar effects are observed in chloroplasts of the filamentous algae Spirogyra, Syragonium and in the unicellular alga Mesotaenium. Dichroism is also observed in the chloroplasts of Spinach and other grana bearing chloroplasts but the scattering depolarization and the small size of the grana in multicellular preparations make quantitative measurements impractical.

Bifluorescence

The bifluorescence of Euglena in the far-red region is an even more striking phenomenon than dichroism. Observation of the polarized emission is most conveniently accomplished using the following arrangement: a Nicol prism (immediately behind the objective) that can be rapidly rotated between two orthogonal positions, a suitable filter to exclude the exciting illumination and an infrared image converter which renders visible the broad banded emission in the 720 mμ region. The observer can thus readily detect the profound changes of fluorescence intensity occuring in the edgewise chloroplasts lying in one or the other of the orthogonal planes of polarization. The infrared image converter when used with aspherical accessory viewing optics is very satisfactory for observations of these kinds and shows very little pin cushion distortion in the center of the field. However, photography of the phosphor screen requires the removal of these correcting optics. Under these conditions the demonstration of the azimuthal designation of bifluorescence in chloroplasts in a field of cells is complicated by off-axis distortion which can give erroneous results. In consideration of this effect and of the limit of resolution imposed by the granularity of the phosphor screen, direct infrared photography was used for these studies and the image converter was reserved for rapid, cursory examinations required for selection of suitable chloroplasts.

Plate I C and D are typical infrared photographs of a group of live Euglena cells showing the characteristics of bifluorescence. The exciting light was unpolarized and consisted of the 436 mμ mercury line and nearby continuum. This, along with the emission in the visual region, was excluded from the photographic film by a Schott RG-9 filter. The open arrows in the figure indicate typical edge-viewed chloroplasts which lie parallel to one of the two orthogonal planes of polarization of the analyzer. The polarized emission is clearly indicated by an increased brightness when the chloroplast lies parallel to a polarization plane and by an attenuated brightness when perpendicular to that plane. Chloroplasts viewed on edge that are positioned with their lamellae at 45° to the polarization planes of the analyzer show identical brightness when observed with the analyzer in either position. Face-viewed chloroplasts on the other hand appear isotropic and show no marked change in brightness at any position of the analyzer. Similar examples of this phenomemon may be found in appropriate chloroplasts within other cells in the figure. The characteristics of chloroplast bifluorescence correspond with those of chloroplast dichroism. Polarized emission as well as polarized absorption is maximal in the direction of the lamellae and indicate a chromophore lying in the lamellar plane. Furthermore, with respect to this chromophore, both types of observation in face-viewed chloroplasts indicate a uniaxial chloroplast structure with the optical axis perpendicular to the lamellae.

R. A. Olson

The environmental factors affecting bifluorescence are similar to those affecting dichroism. Chloroplasts in damaged cells and chloroplasts extruded into aqueous media do not show bifluorescence. Likewise, in chloroplasts subjected to chlorophyll photooxidative bleaching by the exciting light, the resulting yellow fluorescence does not exhibit polarized emission. A dependence of the polarization phenomena on the integrity of the chloroplast structure at the molecular level is indicated.

Chloroplast bifluorescence is indifferent to the polarization plane of the exciting light used. Regardless of the vibration plane of the exciting light the direction and intensity of polarized emission of each chloroplast in the specimen field remains unchanged. This confirms that the oscillators of absorption are not identical to those of emission and that, during the excited state, energy is transferred by inductive resonance from unoriented absorbing chromophores to oriented emitting chromophores. A similar energy transfer has been shown to occur between chlorophyll and accessory pigments and a far-red absorbing form of chlorophylla *in vivo*.[10]

Spectral measurements of bifluorescence in *Euglena* chloroplasts involve even more rigid technical requirements than those of dichroism. Since the quantum efficiency of chlorophyll fluorescence *in vivo* is very low, an intense exciting light in 436 mμ region of high absorption for chlorophyll is required to produce sufficient emission for measurement from a small chloroplast area. A scanning microspectrophotofluorimeter can be used to advantage for spectral measurements of chloroplast bifluorescence.[14] The device requires no other modification than (1) a means of observing the position of the chloroplast relative to the entrance slit of the monochromator and (2) a provision for measuring emission spectra polarized in each of two orthogonal planes. Chloroplasts which showed a maximum difference of emission in the two orthogonal planes of polarization were chosen for measurement. Typical oscillograph traces for the emission form an edge-viewed *Euglena* chloroplast are shown in Plate II A. The upper trace records the spectral distribution of emission transmitted by the Polaroid with its plane of polarization parallel to the lamellar plane, while the lower trace gives that perpendicular to the lamellar plane. Plate II B shows the results of similar optical measurements of the fluorescence emitted from an adjacent face-viewed chloroplast. Quantitative comparison of the two measurements is denied by differences in the actual area of chloroplasts observed. It is clear, however, that in edge-viewed chloroplasts, the maximum polarized emission with the electric vector parallel to the lamellar plane is nearly 50% greater than that with the electric vector perpendicular to the lamellar plane. In face-viewed chloroplasts the effect is insignificant. It is also clear that the maximum bifluorescence occurs in the 720 mμ spectral region. The data from Plate II A are plotted in Fig. 3. Bifluorescence is plotted as a difference, at each wave length, of the emission in the two orthogonal planes of polarization. No correction has been made for the spectral sensitivity of the S-1 photocathode. Such a correction would enhance the values for emission in the shorter wave lengths. Similar measurements were carried out for edge-viewed *Mougeotia* chloroplasts, Fig. 4. The effect, while somewhat reduced, shows similar spectral characteristics with maximum bifluorescence near 720 mμ. The dichroism and bifluorescence

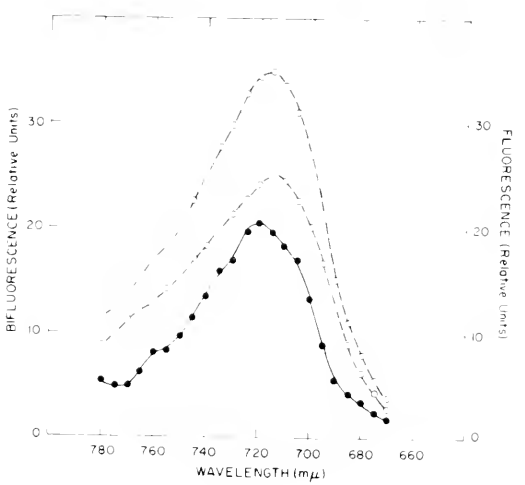
BIFLUORESCENCE (Relative Units)
FLUORESCENCE (Relative Units)
30
20
10
0
780
760
740
720
700
680
660
WAVELENGTH (mμ)

Fig. 3

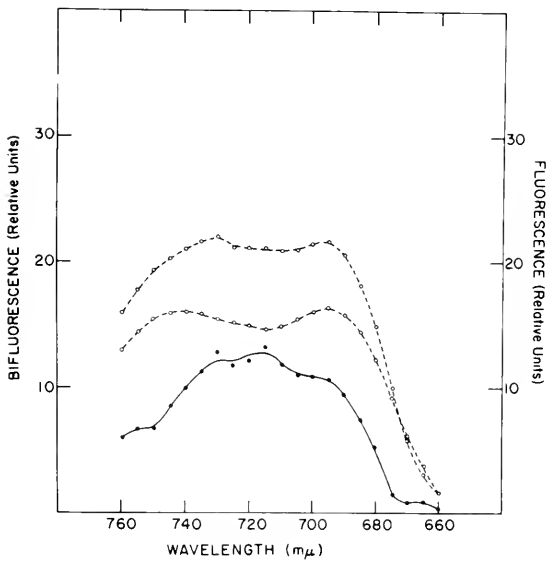
BIFLUORESCENCE (Relative Units)
FLUORESCENCE (Relative Units)
30
20
10
760
740
720
700
680
660
WAVELENGTH (mμ)

Fig. 4

measurements offered in this presentation indicate orientation of a far-red absorbing form of chlorophyll. In addition they suggest strongly the radiationless transfer of energy from randomly oriented oscillators absorbing at shorter wave lengths.

Confirmation of this energy transfer could be obtained if direct excitation of the oriented oscillators by polarized light demonstrated a dependence of the polarized emission on the plane of polarization of the exciting light. In this way the transfer of excitation energy from successive random oscillators absorbing at shorter wave lengths would be by-passed. In this regard we would like to present briefly some preliminary data to support a concept of energy transfer to oriented chlorophyll. It was pointed out above that, since the quantum efficiency of fluorescence in vivo is very low, a high intensity source of exciting light is required for fluorescence microscopy of chloroplasts. No convenient high pressure arc source is available with its output concentrated in the absorbing region of oriented chlorophyll, other means of excitation of its oscillators must be sought. The pulsed ruby laser meets these requirements most appropriately. It has almost unlimited power and an exceedingly narrow band pass at 694.3 mμ, a wave length very close to the region of maximum absorption of oriented chlorophyll. The narrow band pass of the ruby laser exciting light facilitates its exclusion from observation at the fluorescence maximum near 720 mμ of oriented chlorophyll. The high intensity of the parallel laser beam eliminates the need of a condenser in fluorescence polarization microscopy and thus avoids depolarization effects introduced by this optical element. Ruby crystals with 90° orientation provide a polarized output thus eliminating the attenuation introduced by the usual polarizer. Joining the laser to the fluorescence microscope required a massive modular optical bench apparatus to be described elsewhere. Observations can be recorded by infrared photography and the laser exciting light removed by an appropriate interference filter having a minimum of "toe" transmission.

Plate I E and F show typical results of laser polarized fluorescence photomicrography with Euglena chloroplasts in vivo. An unconcentrated laser beam provided the exciting light. The ruby was oriented with the electric vector of the linearly polarized beam as indicated by the arrow labelled "E." It is clear from the photomicrographs that maximum polarized near infrared emission in edge-viewed chloroplasts has its electric vector parallel to that of the polarized exciting light. The direct excitation by polarized light of the 694 mμ absorbing oscillators oriented in the lamellar plane results in a fluorescence polarized in the same direction as the exciting light. The emitted light suffers little depolarization, and the oscillators of absorption appear to be identical to the oscillators of emission.

While observations like those in Plate I E and F serve to localize and designate this polarization property with respect to the chloroplast lamellar plane, they are not appropriate for quantitative purposes. Quantitative measurements of the degree to which the polarized fluorescence retains the sense of polarization of the exciting light can be made more conveniently in cell suspensions. Application of the classic experiments of Perrin and others on the so-called

"depolarization of fluorescence" to chloroplasts has been effected by Arnold and Meek.[15,16]

Difficulty in excluding scattered exciting light limited their measurements to the spectral region below 630 mμ region. The low polarization of fluorescence from the chloroplasts of living cells was attributed to the fact that those chlorophyll molecules which emit fluorescence did not themselves absorb the exciting light. The energy transfer thus demonstrated is identical to that indicated by our study, namely: the polarized infrared emission of chloroplasts is independent of the plane of polarization of the exciting light.

The depolarization of fluorescence of a dilute Euglena suspension was measured using the polarized ruby laser as the excitation source. Typical results from such measurements are shown in Plate II C. The upper oscillograph trace shows the time course of I_{11} during the laser flash; that of $I_{\perp}$ is indicated by the lower trace (horizontal oscillograph deflection 100 microsec/cm). A preliminary calculation of the degree of polarization of fluorescence for the curve maxima indicates a high degree of retention of the sense of polarization of the exciting light. It further indicates that the absorbing oscillators (694 mμ) and the emitting oscillators (720 mμ) are coincident.

DISCUSSION

The evidence presented indicates an oriented far-red pigment which is assumed to be an energy trapping form of chlorophyll a. The red oscillator (~705 mμ), located in the plane of the porphyrin head, appears to be highly aligned parallel to the chloroplast lamellae. This oscillator emits fluorescence (720 mμ) highly polarized parallel to the lamellar plane. If this pigment is a chlorophyll, the absence of dichroism in the blue oscillator indicates that the plane of the porphyrin head does not lie parallel to the lamellar plane. It would also be of value to determine the molar concentration of this pigment in vivo in relation to other pigments involved in order to consider turnover rates, fundamental unit size, and other factors at the site of light transformation.

While the spectral region defined by the maximum dichroic effect indicates in general the absorption characteristics of the oriented chlorophyll it should be noted that actual absorption spectrum is not clearly defined. The absorbing system as a whole in chloroplasts is composed of both oriented and unoriented components each with dissimilar absorption characteristics which spectrally overlap. All of the absorption, for example, at 705 mμ is not due to the oriented pigment. Likewise, within the limits defined by high resolution polarization microscopy the absolute degree of orientation of oscillators cannot be determined. Higher dichroic ratios would have been obtained in an ideal optical system devoid of depolarization effects. However, the approximate definition of the absorption and emission characteristics of oriented chlorophyll by spectral measurements of dichroism and bifluorescence may relate this pigment with C_{705} (Butler), P_{700} (Kok), and Ca_{695} (Brown and French).[10,17,18] The demonstrated ability of oriented chlorophyll to accept excitation energy from other unoriented

oscillators absorbing at shorter wave lengths may also be identified with the inductive resonance energy acceptance behavior C_{705}.

It appears obvious that an oriented pigment should be related to the orderly fine structure of the chloroplast lamellae. The interdependence of chlorophyll and protein in the chloroplast structural array has been demonstrated by 1) the dependence of lamellar development on chlorophyll formation and 2) the destructive effect of proteolytic enzymes on the lamellar pigment structure.[19-21] In the chlorophyll-protein complex implied by these and other investigations it would be expected that molecular orientation of pigment would involve molecular orientation of the attached macromolecule. Therefore it should be the orientational aspects of the protein moiety in chloroplasts that command attention rather than those of chlorophyll alone. Dimeric or trimeric forms of chlorophyll contributing to orientation must be compatible with attachment sites in the protein. Our present knowledge of the nature of chloroplast protein is quite limited. While the amino acid composition has been recently determined, the sequence is unknown.[22] Moreover, the polypeptide configuration of the chloroplast protein has not been designated.

Polypeptides can be molecularly oriented in mesophases. In this regard it is of interest to examine some of the mesophase properties of synthetic polypeptides. Robinson has studied the intrinsic ability of poly-γ-benzyl-L-glutamate to form, spontaneously, lamellar structures.[23] The remarkable likeness of such structures to those in chloroplasts is shown in Plate II D. The lamellar spacing depends on concentration, solvent, temperature, etc., but has a reproducible value for any one set of conditions. It may be as large as 100 μ or too small to be resolved by visual microscopy. Optical anisotropic properties of this lamellar structure indicate it to be a uniaxial system with the optical axis perpendicular to the plane of the lamellae. The optical properties of this structure thereby simulate those of the chloroplast. Optical rotatory measurements indicate the lamellae to be composed of long, parallel helices with a periodicity of rotation of the direction of orientation in successive lamellar planes. By analogy, chloroplast lamellae would be composed of long, parallel polypeptide helices and the orientation of chlorophyll molecules would be governed by available attachment sites. Attempts in our laboratory to adsorb chlorophyll on such mesophase structures were unsuccessful. The polymer is extremely hydrophobic and the solvents required for its structural development are such excellent solvents for chlorophyll that the pigment resists adsorption. It might be expected that a similar structural array of native, long hydrophilic polypeptide molecules in chloroplasts could be detected optically by measurements of dichroism in the ultraviolet region. Ruch, however, was unable to detect any ultraviolet dichroism in fixed and extracted chloroplasts and concluded that a globin or spherical protein molecule was involved.[24] In our laboratory we have been unable to detect ultraviolet dichroism in the chloroplasts of living Euglena cells. These observations however, do not automatically preclude a polypeptide helix array in the chloroplast. Perhaps the employment of more refined observation techniques would detect such optical effects in the chloroplast.

Another insight into the nature of the configuration of tetraporphyrin plane in protein is offered by the hemoglobin macromolecule. In this molecule both sequence and configuration of amino acid residues have been established as well as the location of the tetraporphyrin, or heme plate.[25] While normal hemoglobin does not appear to be molecularly organized, a genetically induced alteration of this molecule causes a molecular phenomenon in erythrocytes known as "sickling". Under conditions of anoxia "sickled cells" become elongated and stretched into a sickle shape by the extension of long chains of stacked hemoglobin molecules. Sickled cells exhibit dichroism in vivo consistent with the absorption of heme chromophores stacked in the direction of cell extension.[26-28] The axis of maximum polarized absorption is perpendicular to the long axis of the sickled cells. This is shown in Plate II E and F taken from a recent paper of Murayama, Olson and Jennings.[28] The plane of the analyzer is indicated by the arrows. An explanation of this selective molecular orientation of tetraporphyrin in such a hemoglobin structure has been offered by Murayama.[29] It involves stacks of the globin molecules which are stabilized by cyclization of the N terminus of the beta polypeptide chains. In the case of Hb-S, cyclization occurs by the formation of hydrophobic bonds and in the case of Hb-C Georgetown by the formation of electrostatic bonds.

The sickled cell thus offers a convincing demonstration of a mesophase or paracrystalline protein structure in vivo in which a tetraporphyrin is molecularly oriented. The relationship of the heme configuration to that of the spherical macromolecule could provide a useful structural analogue to the chloroplast pigment complex. Much more information regarding the structure of chlorophyll protein in vivo (amino acid sequence, electron density distribution, etc.) will be required before similar sub-molecular aspects of chlorophyll molecular orientation are resolved.

Finally, we must consider the significance and virtue, if any, of oscillator orientation at the site of chemical transformation of radiant energy. The relation between organization and specificity has not yet been established in biological systems. A structurally dependent biochemical system in mitochondria has been proposed by Green.[30] A structural array of enzyme molecules is implied, featuring fixed positions in space located to facilitate successive reactions. Such "solid state enzymology" has been disputed by Dixon and Webb.[31] (The subject is discussed by Lehninger and by Kauzmann[32,33]).

Perhaps the organization of molecules for specific reaction is limited to photochemical sites of energy transformation. Pronounced molecular orientation occurs in the visual apparatus. Liebman has measured in vivo dichroic ratios as high as 6.0 in the lamellar structure of the retinal rod outer segment.[34] Similarly, Jaffe has demonstrated a pronounced orientation of the oscillators responsible for photomorphogenesis in Fucus egg cells.[35] An intensive search for other more subtle properties common to these systems and oriented chlorophyll may well resolve the role of oriented oscillators in photobiological systems.

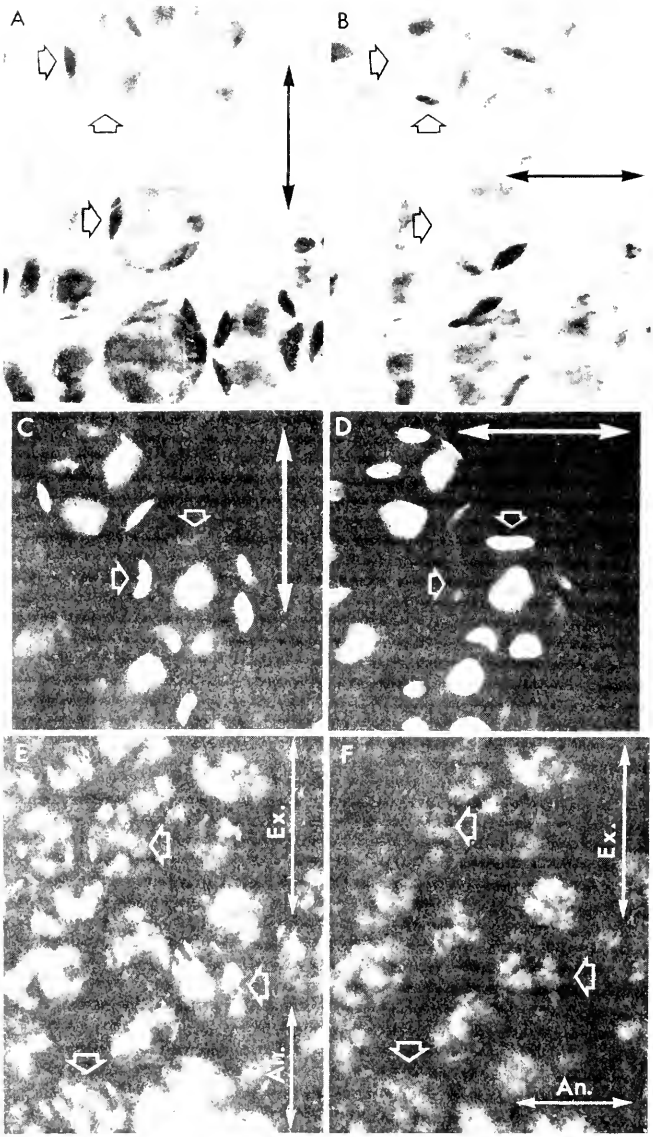

Plate I—A and B: Dichroism of Euglena Chloroplasts at 695 mμ; C and D: Bifluorescence of Euglena chloroplasts excited by unpolarized 436 mμ and observed at 720 mμ; E and F: Bifluorescence of Euglena chloroplasts excited by polarized ruby laser light (694.38 mμ) and observed at 720 mμ.

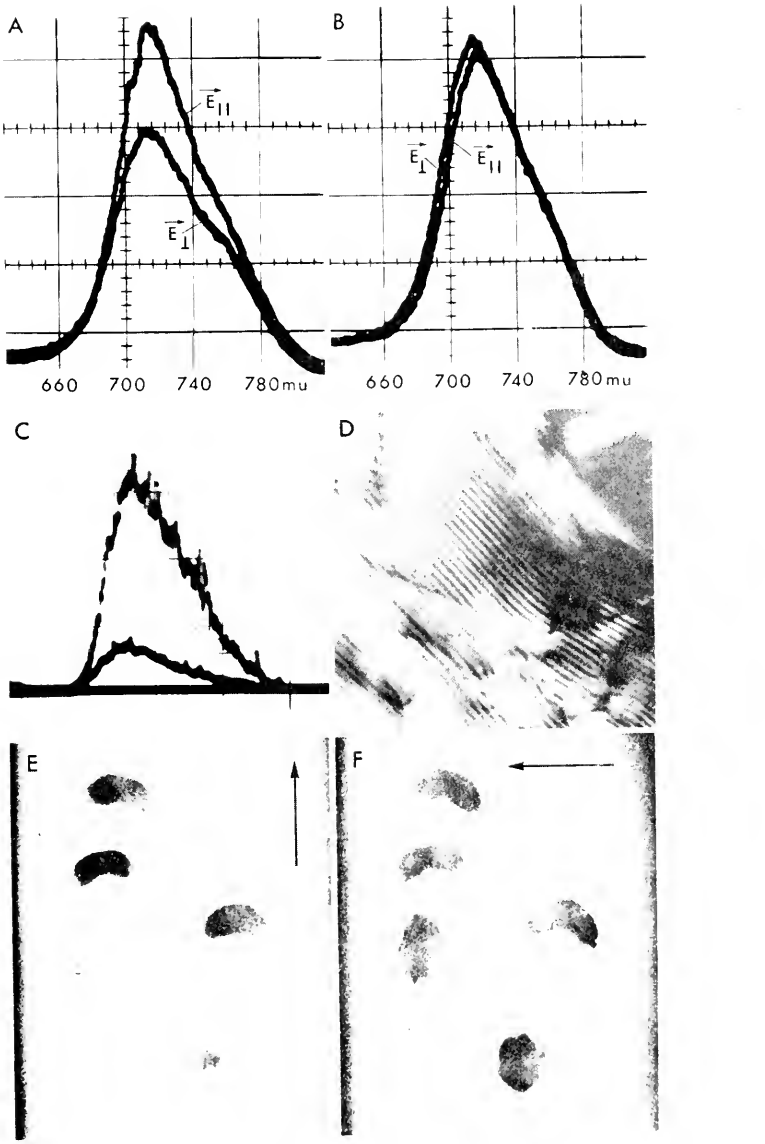

Plate II—A: Oscillograph display of the spectral dependence of Euglena bifluorescence when the chloroplast is viewed in profile; B: The same as II A except the chloroplast is viewed in the facial aspect; C: Depolarization of fluorescence (720 mμ) see text; D: Mesophase of poly-benzyl-L-glutamate in dioxan (after Robinson, 1961); E and F: Dichroism of sickled erythrocytes (Hb-C Georgetown) 435 mμ.

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STUDIES WITH CYANIDIUM CALDARIUM. II. THE FINE STRUCTURE OF PIGMENT-DEFICIENT MUTANTS

Lawrence Bogorad, Frank V. Mercer, and Rosemary Mullens

The complete understanding of photosynthesis requires knowledge about physical and biochemical events in relation to the structure of the photosynthetic system. The early suggestion of Hubert[1] that the chloroplast contains alternating layers of protein and lipid --- with chlorophyll associated with both layers and the carotenoids embedded in the lipid phase --- has served as a model for most subsequent speculation and work[2,3,4] on the molecular structure of the photosynthetic system.

Electron microscopy has confirmed the existence of a multilayered structure within chloroplasts. This lamellar system is differentiated into grana and intergrana lamellae in most chloroplasts of higher plants; chloroplasts of algae and some other species consist of simple lamellae only[2,5].

There is considerable evidence that the photosynthetic lamellae consist of protein-lipid layers; that the chlorophyll may be tightly bound to protein[4,16]; that the other photosynthetic pigments are closely associated with the chlorophylls[7]; and that the pigments have a key role in the photochemical activity of the lamellae[8]. Because of these points it seems reasonable to suppose that, in addition to their photosynthetic roles, the pigments may also be essential structural constituents of the photosynthetic lamellae, and have a casual role in the morphogenesis of the lamellae[17,18]. In the present paper these possibilities are considered in relation to the structure and orientation of the lamellae in non-grana type chloroplasts.

EXPERIMENTAL

Lamellae of non-grana chloroplasts

The dimensions of the photosynthetic lamellae of non-grana type chloroplasts of the higher plants measured on electron micrographs show that the basic structural unit of lamellae is the

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unit membrane structure[2,8].

Cytoplasmic photosynthetic lamellae

Some photosynthetic pigments of the blue-green algae are associated with lamellae[9] and after permanganate fixation, these lamellae appear to be two unit membranes in close contact, the dark bands measuring 20 Å and 40 Å in width and the light bands 35 Å in width[10]. Measurements made on six blue-green genera have confirmed this basic pattern of the cytoplasmic lamellae, but the dimensions of the outer dark and light bands are ca.30 Å and the inner dark band 60 Å[11]. These dimensions, therefore, are very similar to those of the photosynthetic lamellae of non-grana type chloroplasts of higher plants. The dark bands have been interpreted to be protein and the light bands to be lipid in association with chlorophyll[10]. On the basis of studies of energy transfer[12,13] the phycobiliproteins are assumed to be located close to chlorophyll *a* in blue-green and red algae[10]. The basic molecular structure proposed for these lamellae therefore is similar to the models proposed for the photosynthetic lamellae of the higher plants[1,2,3,4].

Photosynthetic lamellae of Cyanidium caldarium

The basic organization of the photosynthetic lamellae in the grana-less chloroplast of *Cyanidium caldarium*, an organism of undetermined taxonomic position, has been shown, by electron microscopy, to be similar to that of other photosynthetic lamellae[14]. *C. caldarium* has been obtained in a number of variously pigmented mutant forms[15]. These mutants, therefore, should offer a way of assessing the importance of the pigments in determining the formation and basic molecular structure of the photosynthetic lamellae. The mutant forms examined by electron microscopy in the present study are described in Table 1.

The cells were grown in a liquid medium[16] with 1 percent glucose at 43 ± 2°C. under fluorescent illumination of 150 to 500 ft-c. Growth was vigorous, and the cells were harvested after 4 or 5 days. For fixation the cells were centrifuged, and the pellet was resuspended in 2 percent buffered $KMnO_4$ fixative pH 7.2 (Veronal acetate, calcium and magnesium chloride 0.001 M, respectively.) After 30 minutes the cells were washed briefly in water before being dehydrated in an ethanol series: 40,70, 100 per cent. The cells were then embedded in methacrylate (75 per cent butyl, 25 per cent methyl, 0.05 per cent benzol peroxide polymerized at 70°C.) Sections were prepared with a Porter-Blum microtome using a diamond knife, and examined in a Siemens Elmiskop

1 at 80 kv. Electron micrographs were taken at 10,000 to 40,000 magnifications.

Table 1
Strains of Cyanidium caldarium studied

Strain		Pigments present
Wild-type	autotrophic	Chlorophyll a, carotenoids, phycocyanin, allophycocyanin.
III-D-2	autotrophic	As in wild-type but greater amounts of all pigments.
III-C*	autotrophic	Chlorophyll a, carotenoids.
GGB	heterotrophic	Phycocyanin, allophycocyanin, carotenoids.
GGB-Y	heterotrophic	Carotenoids.

*Cells contain approximately 40% less chlorophyll a than wild-type. Extracts of III-C cells do not react with antibody prepared by immunizing rabbits with C. caldarium phycocyanin. Therefore, this mutant most likely lacks the protein as well as the bile pigment of phycocyanin.

As can be seen from Figures 1,2,3,4,5 in all the types shown the individual photosynthetic lamellae have the same basic pattern consisting of two unit membranes in close contact. Occasionally, in some sections, the unit membranes had separated over short distances, showing clearly that the lamellae consist of two unit membranes in close contact. The dimensions of each unit membrane are similar to those that have been described for the photosynthetic lamellae of the blue-green algae and certain higher plants. Each unit membrane consists of two dark bands, approximately 25 Å - 30 Å wide, separated by a light band approximately 30 Å wide. The structure of the unit membrane appears to be independent of pigment composition. There are, however, striking differences in the arrangement and amount of lamellar system, and in chloroplast organization among the mutants examined (Figures 1,2,3,4,5).

In III-D-2, wild-type, and III-C strains, the mature chloroplasts are well formed, of similar sizes, and enclosed by a distinct limiting membrane. The main difference appears to be in the number of lamellae present. Of these three strains, cells of mutant III-D-2 (Figure 1) contain the highest concentration of all three pigments; they also have the greatest number of lamellae per chloroplast and the most highly organized chloroplasts. Mature cells of wild-type (Figure 2) closely resemble those of III-D-2, but their chloroplasts have fewer lamellae and

a slightly more open organization. Chloroplasts in mature cells of III-C (Figure 3), a strain which lacks phycobiliproteins, are only slightly less organized than those of III-D-2 and the wild-type.

Considerable variation in the orderliness of the lamellar system is observed in these three cell types. This may be related to the angle of sectioning or the age of the cells; a breakdown of structure has been observed in cells of old cultures. (Prior to division a complete disorganization of the lamellar system and chloroplast also occurs, but these changes are not relevant to the present discussion.) Thus, from a comparison of III-D-2, III-C, and wild-type cells, it seems that phycobiliproteins are not essential structural elements of the photosynthetic lamellae, nor are they necessary for the overall organization of the lamellar system of the mature chloroplast.

In mature cells of mutant GGB, a strain which lacks chlorophyll a but contains phycobiliproteins, the lamellae have a unit membrane structure similar to that in III-D-2 and wild-type (Figure 4c) but they are usually shorter --- a few hundred Å to about a micron in length. In general the lamellar system is less well organized than in the other strains described (Figure 4): In the peripheral region of the chloroplast, the lamellae are frequently arranged in parallel rows of about the same length as in wild-type and occasionally (Figure 4b) most of the lamellae show parallel arrangement. Usually in the main body of the chloroplast, however, the lamellae are much shorter. The smaller pieces tend to be scattered at random, while the larger pieces tend to be parallel but with a wavy orientation. The total amount of lamellae, as represented by the profiles of the segments in the sections, is much less than in wild-type or III-D-2, possibly less than a quarter. In some sections large areas of the chloroplasts contain only a few short lamellae. The mature chloroplasts are similar in size to those in wild-type, but have a more irregular shape. The limiting membrane is not always distinct and tends to merge with the cytoplasmic membranes. Because of this, the chloroplasts in GGB are not as clearly differentiated from the cytoplasm as in III-D-2, wild-type, and III-C.

The mutant GGB-Y has a structure similar to GGB, but its chloroplast is much less organized (Figure 5). The chloroplasts are irregular in shape; the limiting membrane is frequently discontinuous so that the lamellae often merge into the cytoplasm.

Thus, the chloroplast in GGB-Y does not appear as a distinct organelle sharply differentiated from the cytoplasm. Rather it resembles a collection of loosely arranged lamellae more or less aggregated into a discrete body; yet in some cells the area occupied by this aggregation is about equal to that of a well organized chloroplast. The individual lamellae have the usual double membrane structure (Figure 5c) but are shorter than in the other mutants, varying from less than a few hundred Å to about a micron in length. Some of the larger lamellae have a parallel orientation (Figure 5b). Usually the orientation is irregular with the lamellae in the peripheral regions often extending into the cytoplasm.

Conclusions

1. The pigments --- chlorophyll *a*, phycocyanin, and allophycocyanin --- do not appear to be essential structural components of the unit membrane.

2. Since the structural integrity of the lamellae does not involve the pigments, it is possible that the pigments normally found in the lamellae may be added to preformed membranes during chloroplast development.

3. The amount of lamellar material and its degree of organization varies among the mutants studied in the following order: III-D-2 > Wild-type and III-C > GGB > GGB-Y. Thus, although pigments are not essential components of the lamellae, their presence affects the extent and organization of the lamellar *system*. It is possible, however, that the low degree of chloroplast organization in GGB and GGB-Y cells may be related to their heterotrophic state. The presence of fewer lamellae and their poor orientation may result from the absence of photosynthetic activity.

4. It has not been possible, from an examination of this set of mutants, i.e. by comparing cells of the wild-type with III-C or GGB with GGB-Y, to determine the normal location of the phycobiliproteins in the chloroplast of *C. caldarium*.

5. Since the unit membrane is the basic structural unit of the cytoplasmic photosynthetic lamellae of the blue-green algae, the photosynthetic lamellae of chloroplasts, and the photosynthetic lamellae of *Cyanidium*, it can be assumed that some of the conclusions reached for this alga are generally applicable. That is, chlorophyll *a* is neither an essential structural component of

"photosynthetic lamellae" nor is it essential for the formation of unit membranes and their association at the level which occurs in C. caldarium in which two or three unit membranes constitute a dense band. However, the orientation of dense bands to one another, the extent of the lamellar system, and the general organization of the chloroplast as a discrete organelle may be affected by the presence of chlorophyll.

Acknowledgements

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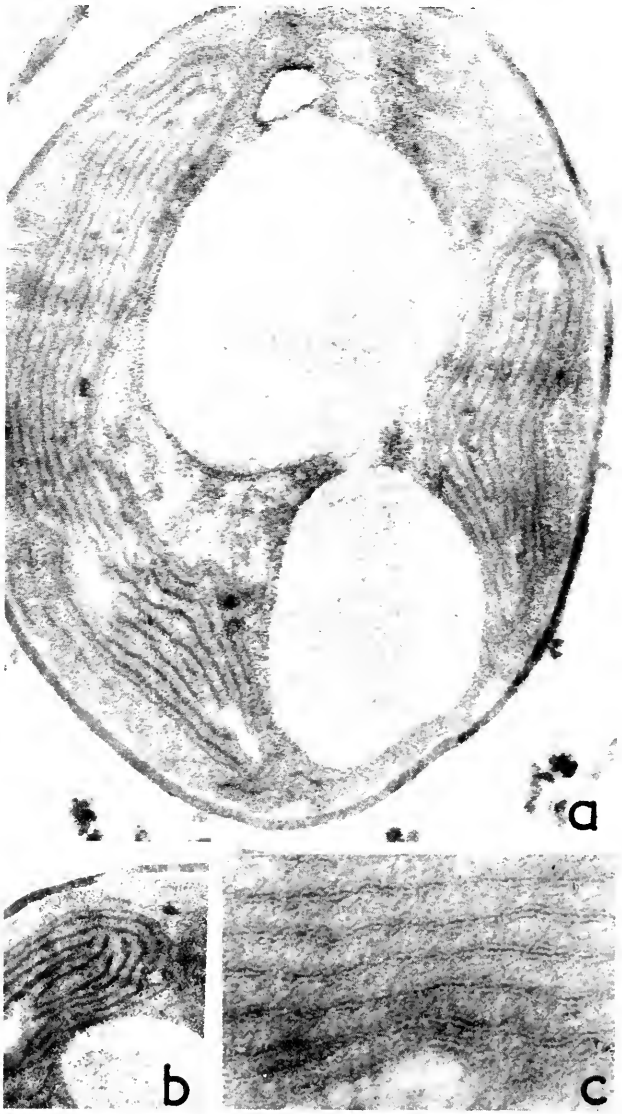

Figure 1. Mutant III-D-2

a) Section of adult cell showing two chloroplast profiles with bands of lamellae enclosed by a limiting membrane. (x 40,000)
b) Chloroplast profile showing lamellae less oriented than in (a). (x 40,000)
c) Section of portion of chloroplast showing bands of lamellae, and double membrane structure. (x 160,000)

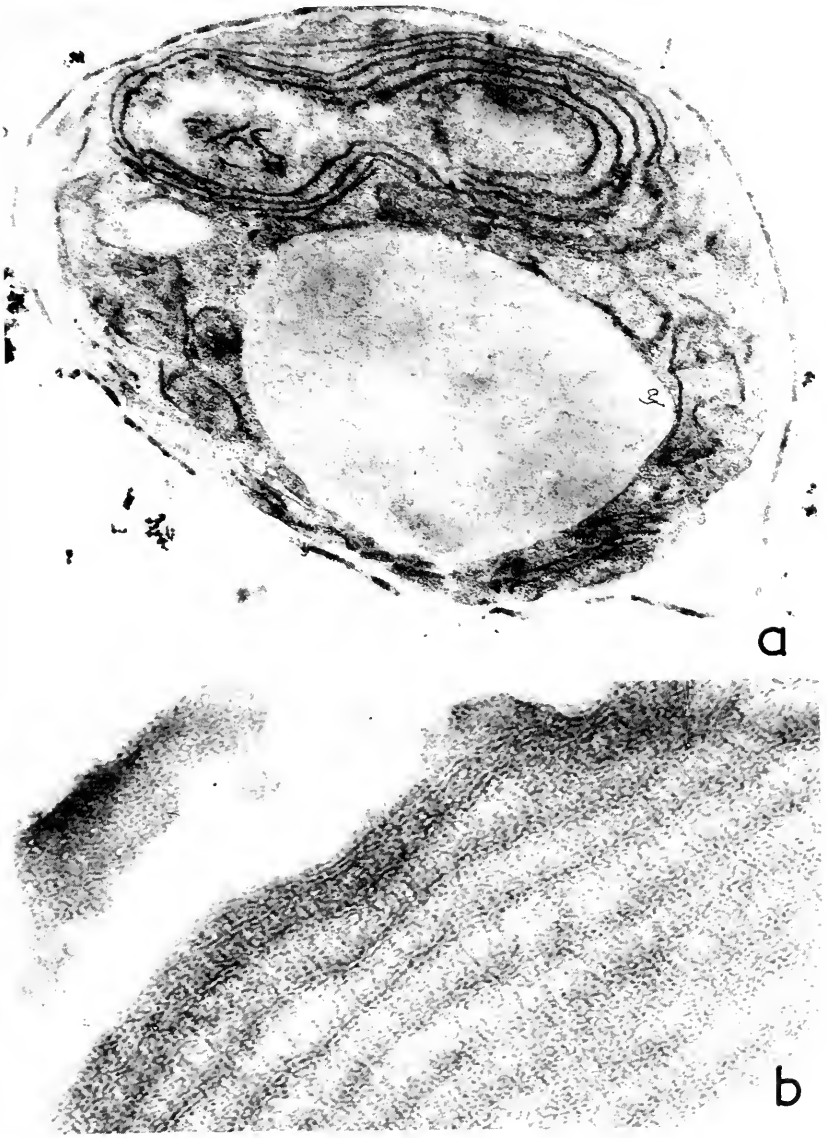

Figure 2. Wild-Type

a) Section of adult cell showing lamellae in the chloroplast. Difference in organization as compared with III-D-2 is due to angle of section - compare Figure 1b. (x 40,000)

b) Section of portion of chloroplast showing bands of lamellae, with double membrane structure. (x 160,000)

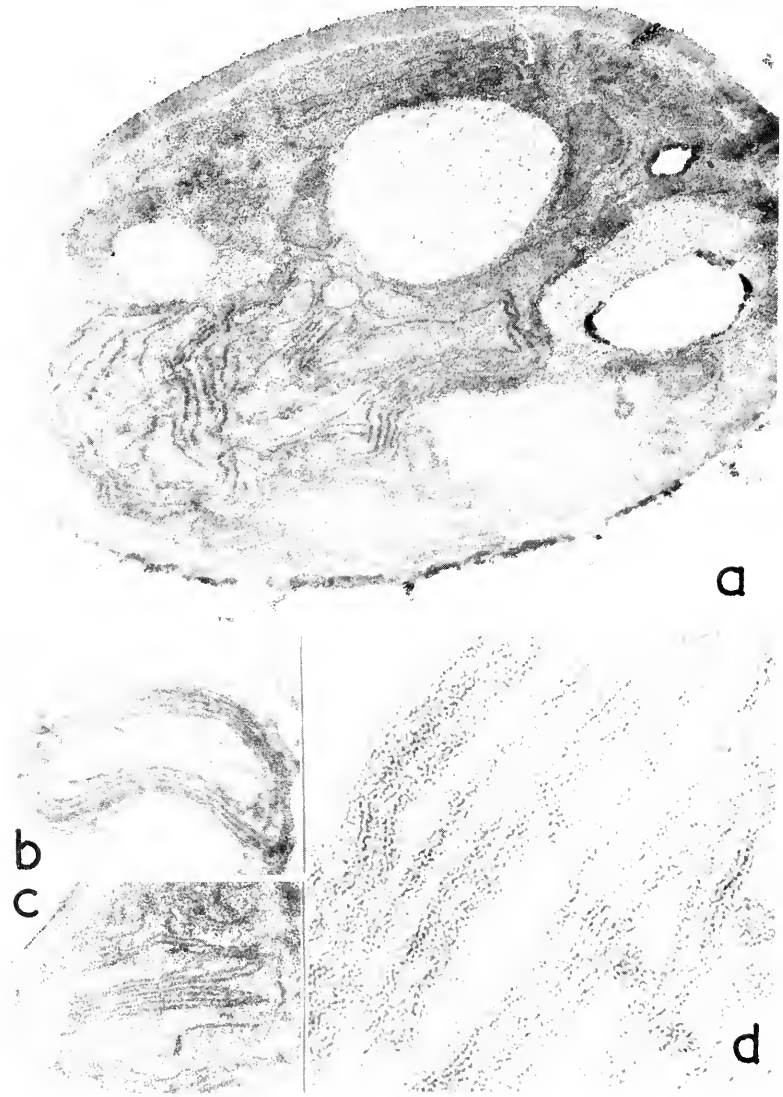

Figure 3. Mutant III-C
(minus phycocyanin and allophycocyanin)

a) Section of cell showing irregular shape of chloroplast. Chloroplast is partially disorganized because the cell is approaching division. (x 40,000)

b) and c) shows parallel arrangement of lamellae and well organized plastids. (x 40,000)

d) Section of portion of chloroplast showing bands of lamellae with double membrane structure. (x 160,000)

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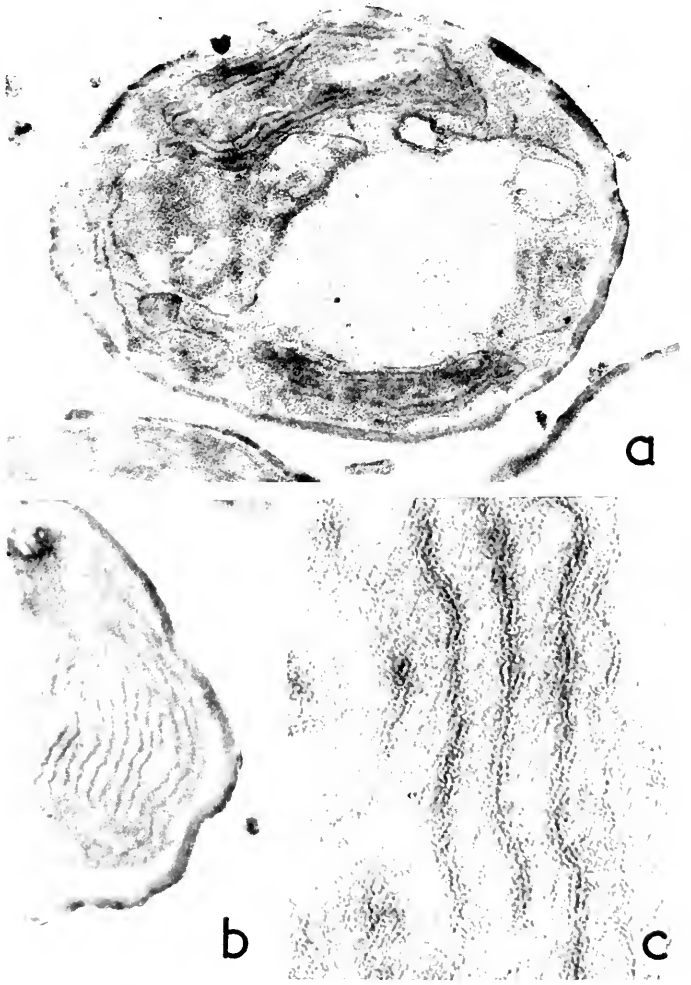

(minus chlorophyll a)

a) Section of cell showing irregular shape of chloroplast. This is normal situation and not due to approaching cell division. (x 40,000)

b) A single chloroplast showing parallel arrangement of lamellae - chlorophyll absent. (x 40,000)

c) Section of portion of chloroplast showing bands of lamellae with double membrane structure.

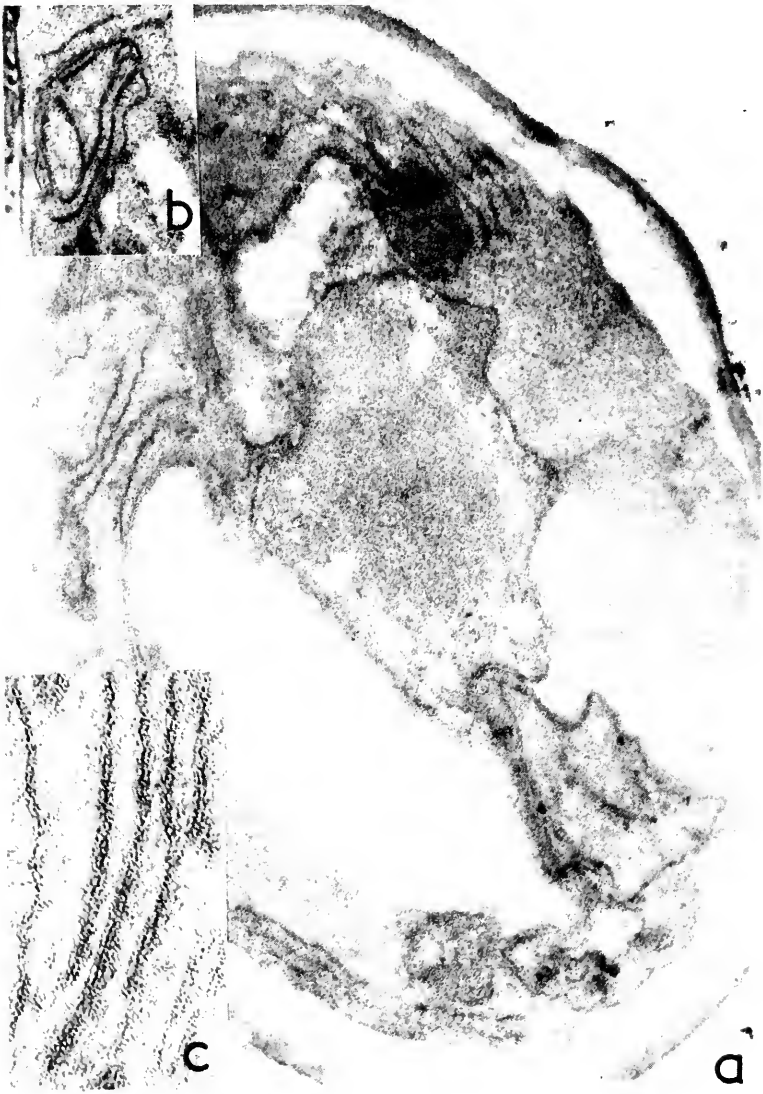

Figure 5. Mutant GGB-Y
(minus chlorophyll a and phycobiliproteins)

a) Section of cell showing poorly organized chloroplast(s). Chloroplast membrane not easily identifiable and lamellae merging into cytoplasm. (x 40,000)
b) Portion of a chloroplast showing that bands of lamellae may have a parallel orientation. (x 40,000)
c) Section of portion of chloroplast showing bands of lamellae with double membrane structure. (x 160,000)

CHLOROPHYLL'S LIPID ENVIRONMENT

A. A. Benson

Estimates[1,2] of the intermolecular distance for chlorophyll in chloroplast lamellae correspond to those of a half-molar solution. Photochemistry of dilute chlorophyll solutions hardly seems pertinent to conditions existing in the chloroplast. Interactions of closely spaced chlorophyll molecules oriented at the water-air interface have recently been reinvestigated[3]. Absorption maxima of the pure pigment were shown to be dependent upon the film pressure, especially when it exceeded the limit of reversible pressure-area relationships. Whether the presence of other highly surfactant molecules might produce similar effects has not yet been demonstrated but appears plausible. The lipids of chloroplasts are among the strong surfactants of Nature.

Myelin Figures

Formation of myelin figures is a characteristic action of certain phospholipids upon hydration[4]. These multilaminar structures exhibit dimensions found in cell organelles. The early observations of Menke[5] and Weber[6] revealed the formation of myelin figures from chloroplast components in intact cells. These birefringent green films were extruded as a result of disorganization of the plastids by a variety of reagents. It is now of interest that the most concentrated lipid of chloroplasts exhibits just such activity when the pure substance is exposed to water[7].

Chloroplast Lipids

The chloroplast lamellar lipids have been identified and recently have been isolated[8]. The glycolipids of Figure 1 and the phospholipids of Figure 2 are the surfactant materials which appear to stabilize the membranes of chloroplasts. These lipids which stabilize membranes of the thylakoids are predominantly amphipathic surfactant substances which, like chlorophyll itself, possess hydrophyllic and hydrophobic groups. The solubility properties and structure of these groups determine the molecules' interfacial adsorption properties and, apparently, the structure and stability of oriented molecular leaflets which occur in the chloroplast.

The hydrophyllic moiety of chloroplast lipids is primarily the α-D-galactosyl-(1→6)-D-galactosyl group of digalactosyl diglyceride. This lipid, together with about half as much of the monogalactosyl diglyceride,

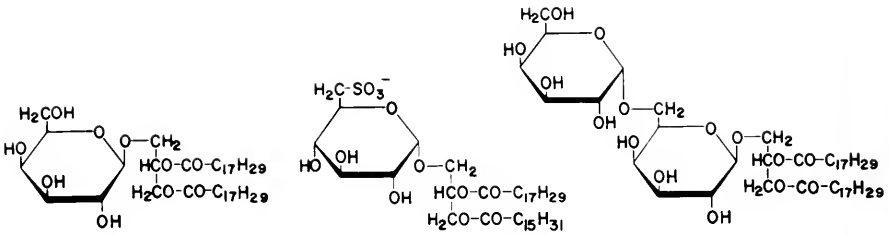

Fig. 1. Glycolipids of chloroplasts. Galactosyl diglyceride, the plant sulfolipid (sulfoquinovosyl diglyceride), and digalactosyl diglyceride.

is the "neutral lipid" to which Menke[9] referred in his early analysis of chloroplast lipids. The water-solubility of the galactosyl groups, coupled with the hydrophobic and liquid properties of the two tri-unsaturated C_{18} fatty esters (linolenic acid), gives these lipids their surfactant properties. They resemble the synthetic non-ionic detergents. The moderate water-solubility of the galactolipids allows one to consider their diffusion or transport in the aqueous phase. Yet, it is not so great as to preclude formation of stable myelin figures when the pure digalactosyl diglyceride is exposed to water.

The most striking aspect of the galactolipids is their phenomenal fatty acid composition. They are the most unsaturated lipids in Nature and contain as much as 96% linolenic acid[10,11,8]. The sulfolipid[8] and phospholipids[11], on the other hand, possess saturated as well as unsaturated acids. Fatty acid compositions of glycolipids isolated from alfalfa and Chlorella are given in Table I.

Table I

Fatty Acid Composition of Glycolipids of Alfalfa and Chlorella[8]

	Monogalactolipid	Sulfolipid	Digalactolipid
Alfalfa	2.7 % Palmitic	42.8 Palmitic	14.0 Palmitic
	95.0 Linolenic	47.2 Linolenic	82.0 Linolenic
Chlorella	2.7 Palmitic	67.5 Palmitic	11.6 Palmitic
	9.7 Palmitoleic	18.3 Oleic	9.5 Palmitoleic
	40.5 Oleic	9.8 Linolenic	36.8 Oleic
	26.8 Linolenic		27.0 Linolenic

A. A. Benson

Lipids of Isolated Chloroplasts

Present estimates of concentrations of the lipid components of plant cell organelles may be affected by enzyme-catalyzed degradation. The lipids of runner bean leaf chloroplasts[12] contained no galactolipids as a result of activation of galactolipases by disruption of the cells. Water homogenates of the leaves catalyzed rapid liberation of fatty acids from pure galactolipids. In other instances we have observed specific degradation of the sulfolipid as a result of breaking Scenedesmus cells[13]. It is possible that inactivation of chloroplasts and their failure to retain certain protein components is a result of lipase action and consequent alteration of the stability of their lipid membranes. Such phenomena are well understood in the case of erythrocyte lysis by phospholipase A.

The unsaturation characteristic of chloroplast lipids leads to the suggestion that the hydrophobic phase of an adsorbed layer will be a liquid rather than possessing the highly oriented structure characteristic of compressed monolayers of saturated fatty acids. This 'liquid' region may be 'dissolved' in hydrophobic sections of adjacent protein molecules. The cytochromes c for which amino acid sequences are known have such hydrophobic regions. It may be found that 'liquid' fatty acid chains like that of linolenic acid are particularly strongly adsorbed by certain hydrophobic regions of proteins.

Chlorophyll's Environment

The phytyl ester moiety of chlorophyll may induce orientation of the molecule to an extent which may approximate that of the glycolipids. This surfactant pigment, then, must be surrounded, in the main, by the predominant chloroplast lipid, digalactosyl diglyceride. The porphyrin, therefore, would be compressed at an interface by hydrated galactose molecules. Since these lipids vary in concentration with illumination one should anticipate changes in association of propinquitous pigments and thereby in their absorption spectra. James Franck suggested that the fluorescent and non-fluorescent forms of chlorophyll are the result of their be situated in aqueous and hydrocarbon phases of the chloroplast. The chlorophyll compressed in a membrane of galactosyl, sulfoquinovosyl, and L-1-glycerophosphoryl groups of the oriented chloroplast lipids. Chlorophyll, more deeply embedded in the hydrocarbon phase of the lipids or adjacent protein would correspond to the non-fluorescing form.

The precise location of the lipid layers which the electron micrographs suggest is still uncertain. The developing understanding of the chemistry and metabolism of these substances promises to provide more probable bases for interpreting present pictorial concepts of chloroplast lamellar structure.[14]

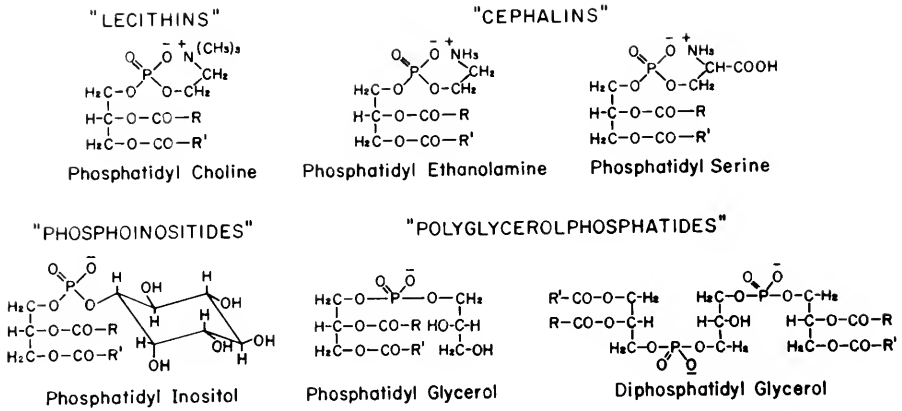

Fig. 2. Phospholipids occurring in chloroplasts. The most concentrated phospholipid in chloroplasts is phosphatidyl glycerol.

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THE CHLOROPLAST STRUCTURE IN PHOTOSYNTHESIS

Jerome J. Wolken

The chemistry and molecular structure of the chloroplast must be linked to its function as an energy capturing, storing, and transferring device in photosynthesis. An organism ideally suited for the study of the chloroplast chemistry and structure is Euglena, which is photosynthetic in the light and "chemosynthetic" in the dark. In these light<--->dark adaptations, biochemical changes are accompanied by structural changes of the chloroplast. In addition, growth at elevated temperatures above 32°C. and drug action (e.g., streptomycin, antihistamines, etc.) interfere with the organism's photometabolism and hence with chlorophyll synthesis, bringing about structural changes of the chloroplast and resulting in chlorophyll-less mutant forms[1].

THE CHLOROPLAST

The precise composition and chemistry of active chloroplasts is still unknown; gross analysis of isolated chloroplasts indicates from 35% to 55% protein, 18% to 37% lipids, mostly phospholipids, and 5% to 8% inorganic material on a dry weight basis. Nucleic acids, RNA and DNA, have been identified, with estimates from 0.3% to 3% on a dry weight basis.[2,3] The chloroplast pigments, the chlorophylls average about 6% and the carotenoids 2%. In Euglena, the chloroplast chlorophylls constitute about 85% chlorophyll a and 15% chlorophyll b [1]. The major carotenoids are β-carotene, lutein, and neoxanthin. However, in Euglena, instead of lutein, 80% of the carotenoids present were identified as antheraxanthin, 11% β-carotene, and 7% neoxanthin[4]. In addition, γ-carotene, crytoxanthin, echinenone, and two new carotenoids, euglenanone and hydroxy-echinenone were identified.

The chlorophyll and carotenoid syntheses can be followed, and their concentrations calculated from the absorption spectra. For example, from such spectra, 1.0×10^9 chlorophyll molecules were calculated for an average chloroplast[5]. It is now possible to obtain the absorption spectrum of a single in vivo chloroplast by techniques in microspectrophotometry[6]. A recorded absorption spectrum of a Euglena chloroplast is illustrated in Fig. 1. Fig.

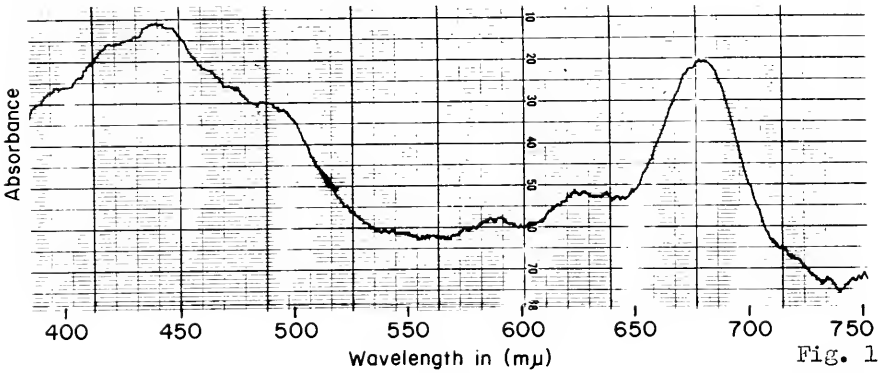


Fig. 1

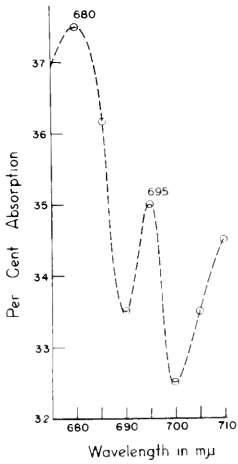


Fig. 2

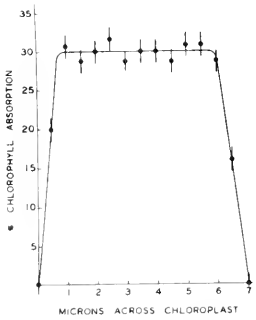


Fig. 3

2 shows fine structure of the spectrum from 675-710 mμ for a very young culture. Chlorophyll a has absorption peaks at 673, 683, and 695 mμ. As the cells age, shifts in the absorption peaks to longer wavelengths occur. Chlorophyll is not a free pigment molecule in the chloroplast, but is complexed to a protein or lipoprotein. The actively photosynthesizing Euglena has chlorophyll uniformly distributed throughout the chloroplast as illustrated in Fig. 3.

Coupled with the chlorophyll-protein of the chloroplast is a cytochrome system. Two spectrally different cytochrome-c types were isolated and characterized from photosynthesizing and non-photosynthesizing Euglena. These are referred to as cytochrome-552 and cytochrome-556[7]. Cytochrome-552 has absorption peaks in the reduced state at 552, 523, and 416 mμ; cytochrome-556 has absorption peaks in the reduced state at 556, 525, and 421 mμ[1]. Cytochrome-556 was also extracted from substrains of HB, heat-bleached and SM, streptomycin-bleached Euglena cells with absorption spectra identical to those extracted from dark-grown Euglena[8].

When dark-grown Euglena are re-adapted to light, during the first 24 hrs., while the cells are actively synthesizing chlorophyll, only cytochrome-556 can be isolated; however, after 48 hrs., only cytochrome-552 is found. The apparent shift from cytochrome-556 to cy-

tochrome-552 is not a result of a non-uniform synthesis of chlorophyll, since sufficient Euglena remained chlorophyll-free during the first 24 hrs. of light exposure to permit the extraction of a high concentration of cytochrome-556. This spectral species of cytochrome must have been isolated from cells in which active chlorophyll synthesis was occurring. The concentration of cytochrome -556 remains relatively constant (4 x 10^7 m moles per gram wet weight of cells); however, cytochrome-552 increases directly with chlorophyll synthesis in a ratio of the order of 1 cytochrome-552 to every 300 chlorophyll molecules. The change-over from one spectral type of cytochrome to the other would be expected to occur earlier than it has been observed. Cytochrome-552 is probably synthesized from a common metabolic pool under the influence of light. Both cytochromes are therefore present in the photosynthetic Euglena.

Manometric measurement of the photosynthetic-respiratory ratio (P/R) shows that until dark-grown Euglena have been illuminated for 24 hrs., the P/R = <1. After this time (at least one generation, varying between 16 and 24 hrs.) the P/R becomes >1 and active photosynthesis can be measured[1]. These data are in agreement with our observations on the increase of chlorophyll and the chlorophyll dependence of cytochrome-552[8].

The oxidation-reduction potential E'_o of cytochrome-552 is +0.38 volts, and for cytochrome-556 is +0.32 volts. These high potentials for the cytochromes indicate that they can take part in the photometabolic electron transport in the chloroplast. However, in view of the measured value of the oxidation-reduction potential for cytochrome-556, which is greater than that expected for the respiratory cytochrome-c as well as for cytochrome-a + a_3 it could not transfer electrons to the oxidase; i.e., it could not be oxidized by the enzyme. It is suggestive, then, that cytochrome-556 is associated with the initial steps leading to chlorophyll synthesis and to the chloroplast structure.[8]

STRUCTURE

To bring the chemistry of the chloroplast together with its structure, a morphological study of Euglena chloroplasts under different environmental conditions has been pursued. In the light microscope, Euglena chloroplasts are easily identified as green organelles (see Fig. 5, a cross-section of a Euglena). Electron microscopy increases the resolution to delineate a chloroplast membrane and internal dense layers (lamellae) with less dense interspaces (Fig. 6). Each dense layer is of the order of 250 Å in

thickness and an average chloroplast contains 21 such dense layers. The dense layer appears to be a composite, a double layer with lamellae from 50 to 100 Å in thickness. Depending on the fixatives used and the resolution of the electron microscope, as many as 4 to 6 lamellae can be delineated (Fig. 6). However, we associate only the two outer lamellae with the chlorophyll molecules. Although chemical analyses of the chloroplast lamellae have not yet been accomplished, the dense layers are taken for lipoproteins and lipids because of their affinity for fixing agents such as OSO_4, and the less dense spaces are aqueous proteins enzymes, and dissolved salts. It was further envisioned that the hydrophilic porphyrin "heads" of the chlorophyll molecules extend into the aqueous protein, and that the lipophilic phytol "tails" reach to the lipid layer.

When Euglena are dark-grown, their chloroplasts are no longer recognizable. These dark-grown Euglena cells contain chloroplast remnants, proplastids[9]. Bleached cells, whether appearing by dark-adaptation, streptomycin, heat, or u.v. light, all possess proplastids. Gibor and Granick[10,11] found that the proplastids of some of these bleached strains were capable of synthesizing porphyrins in a standard culture medium. Others synthesized porphyrins only after the addition to the medium of delta-aminolevulinic acid, a precursor to porphyrin synthesis[11]. Upon re-adaptation to light, the chloroplast development can be followed at regular time intervals. After as little as 4 hrs. in the light, elongated bodies characteristic of chloroplast lamellae appear, but these are thinner and not regularly packed. The lamellae, few in number at first, increase progressively with time in continuous light, and by 72 hrs. (3-4 generations under our conditions) the chloroplasts have the shape and lamellar organization described for active photosynthetic Euglenas grown in light for many generations (Figs. 5 and 6). The number of lamellae that are formed can be correlated with chlorophyll synthesis, and to the number of chlorophyll molecules[1,5]. This kind of structure has the advantage of bringing a large surface area necessary for the number of chlorophyll molecules in the chloroplast to be compressed into a small volume.

To see how much chlorophyll was available to the chloroplast, and what area of the total lamellar surfaces would be occupied by the chlorophyll molecules, the chlorophyll concentration per chloroplast was determined and the number and thickness of dense layers was statistically evaluated (Table I). The validity for monomolecular layers of chlorophyll molecules on the surfaces of the chloroplast lamellae was determined by calculating the area avail-

able for the porphyrin of the chlorophyll molecule[5]. This was found to be 222 $Å^2$, which is about the right cross-section for a porphyrin molecule spread on a water-air interface. Similar calculations since made for the chlorophyll cross-sectional area in the chloroplast of a variety of plants were also found to be of the order of 200 $Å^2$ [12,13].

TABLE I

Chloroplast Euglena gracilis

diameter	1.23 μ (1.04 - 1.42)
length, D	6.50 μ (5.2 - 9.3)
number of dense layers, n	21 (18 - 24)
dense layer thickness, T	242 Å (180 - 303)
interspace layer thickness	374 Å (300 - 476)
chlorophyll molecules, N	1.02×10^9 (0.88 - 1.36)

Mean and Extremes in the Measurements.

On the basis of the chlorophyll concentration, structural data, and calculations of the area and volume occupied by the pigment molecules of the chloroplast, the schematic molecular model illustrated in Fig. 4 was proposed[5]. The model (Fig. 4a) includes the suggestion of Baas Becking and Hanson[14] that 4 chlorophyll molecules are united to form tetrads in such a way that only one of the phytol tails is located at each intersection in the rectangular network. This arrangement has the advantage of leaving adequate space for the carotenoid pigments. If these spaces are occupied as illustrated, there would be at least one carotenoid molecule for every three chlorophyll molecules in the network. Since the molecular weights of the carotenoid molecules are one-half to two-thirds of the molecular weight of the chlorophyll molecules, a weight ratio , chlorophyll to carotenoid, of approximately 4:1 to 6:1 would be expected. On the other hand, the carotenoid molecules are slender linear molecules, probably 5 Å in diameter, and therefore more than one molecule could conveniently fit into the 15 Å x 15 Å holes formed by the chlorophyll tetrads. From symmetry, one might expect as many as four molecules per hole, but this would lead to very tight fitting, which would be energetically improbable. One can therefore put a lower limit on the number of chlorophyll to carotenoid molecules of roughly 1:1, and a weight ratio of 2:1. The close packing of the chlorophyll and carotenoid molecules in the pigment monolayers of the chloroplast could permit energetic interaction between the pigment molecules. The average number of chlorophyll molecules in the interfacial layers is obtained by simply dividing the chlorophyll con-

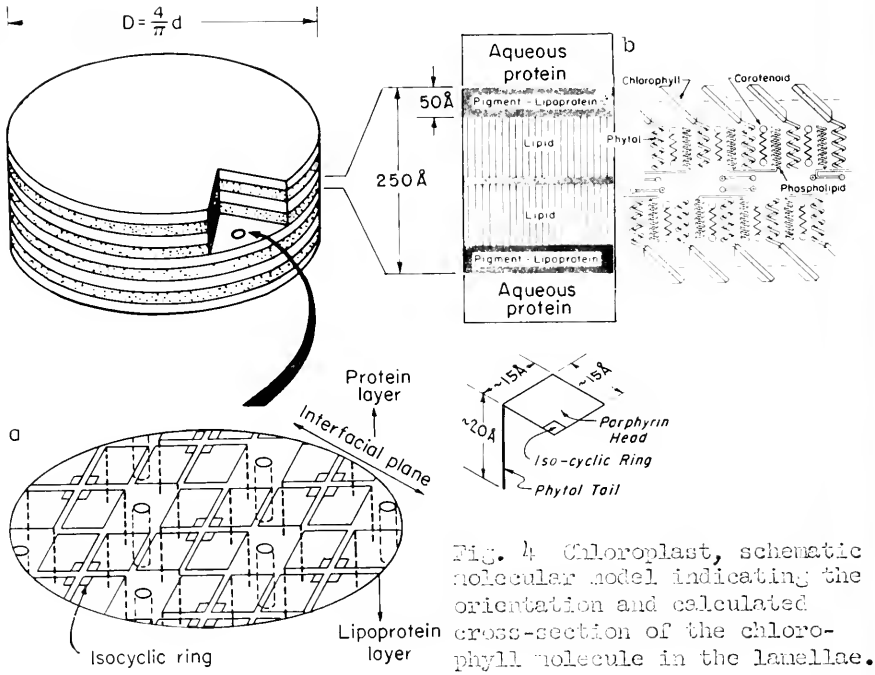


Fig. 4 Chloroplast, schematic molecular model indicating the orientation and calculated cross-section of the chlorophyll molecule in the lamellae.

centration (1×10^{9}) by twice the number of dense layers; hence the number would be 25×10^{7} molecules per layer. The number of chlorophyll molecules per unit area in the interfacial monolayers is just the reciprocal of the area available to each molecule, i.e., approximately 4×10^{13} per cm^2.

There are several possible ways in which the chlorophyll molecules could be oriented in the chloroplast lamellae. If the porphyrin heads of the chlorophyll molecules lie at 0^{o} as flat plates as depicted in Fig. 4a, their greatest cross-section would be available. However, if they are oriented at increasing angles up to 90^{o}, the cross-sectional area available would be decreased. Studies of chlorophyll monolayers at various liquid surfaces suggest that the chlorophyll molecules probably lie at angles from 25 to 55^{o} within the lamellae, thus reducing our calculated cross-section of the chlorophyll molecule to about 100 $Å^2$. Chlorophyll-a has been shown to crystallize out in thin sheets of 50 Å, corresponding to two molecular layers. Since the crystallized chlorophyll molecules occupied an area of 106 $Å^2$ (15), the porphyrin "head" of the chlorophyll molecule could be tilted at an angle near 45^{o}. However, the chlorophyll molecules in the living cells

are in a dynamic state, and are arranged so that their absorption oscillators are oriented for maximum absorption.

THE CHLOROPHYLL-PROTEIN

The molecular weight of the chlorophyll-protein in the chloroplast can be estimated from the chloroplast structural data (Table I), in which the geometry and chlorophyll concentration of the chloroplast is employed. The assumption is that the electron-dense layers would contain a double layer of protein macromolecules, and that there is one chlorophyll molecule per protein molecule. An equation can be written in a form containing only the experimental measurements:

$$M = \frac{\pi D^2 TsLn}{4N}$$

where D is the length, T is the thickness of the dense layers, s is the density taken as 1.3 that of a protein (if a lipoprotein, the density would be closer to 1.1), L is Avogadro's number, n is the number of dense layers, and N is the number of chlorophyll molecules. The molecular weight calculated from this equation is of the order of 21,000[16]. The molecular weight was also estimated from data taken from the in vivo Euglena chloroplast using interference microscopy, the area of the chloroplast, and its chlorophyll concentration. The calculated molecular weight obtained was 16,000[1,16]. Frey-Wyssling[17] suggests that the lamellae are globular macromolecules of the order of 65 Å in diameter (see Fig. 7), which would accomodate 16 chlorophyll molecules with a molecular weight of the order of 68,000. These calculations give us an estimate of the minimum molecular weight of a chlorophyll-protein molecule.

CHLOROPLASTIN

Since the chloroplasts contain large quantities of lipid, it is difficult to solubilize them in water. However, colloidal suspensions of the chloroplasts can be prepared by ultrasonic techniques and by extraction with detergents (digitonin, tergitol). Detergents in solution form colloid aggregates, micelles, that have a very strong attraction for many of the more complex dye molecules. The non-ionic recrystallized digitonin ($C_{55}H_{90}O_{20}$) a digitalis glycoside, is a nitrogen-free detergent, and has a cholesteric structure. Digitonin because of its properties, opens the pigment-lipid, lipoprotein layers of the chloroplast and forms chloroplastin micelles[18]. Sedimentation in an analytical ultracentrifuge, and electrophoresis, show that chloroplastin behaves as a homogeneous micelle system[15]. Absorption spectra of

chloroplastin shows similarities to the absorption spectrum of a chloroplast. However, chloroplastin is a very complex system of chlorophyll, carotenoid, cytochrome, phospholipid, lipids, and protein (Table II).

TABLE II
Chloroplastin

w, dry weight mg/ml	27.3
n, nitrogen mg/ml	0.36
p, chlorophyll moles/ml	6.3×10^{-5}
c, cytochrome-552 moles/ml	1.8×10^{-7}

Not included in this analysis are the carotenoids which are also extracted.

Chloroplastin, not fixed or stained, and viewed directly in the electron microscope (Fig. 8) reveals particles (<100-1000Å) of the same order as observed by electron microscopy in the fixed chloroplast sections (Fig. 7). If each chloroplastin micelle is estimated to be about 200 Å in diameter, it would have 225 chlorophyll molecules, 55 carotenoid molecules, and one cytochrome molecule with its protein and lipids, and its molecular weight would be of the order of a million. This is in agreement with the "chlorophyll holochrome" of Smith[19] and close to the minimum size for the active photosynthetic unit as postulated by Emerson and Arnold[20].

PHOTOCHEMICAL ACTIVITY

Chloroplastin exhibits photochemical activity in solution. The photoreduction of the dye 2,6, dichlorobenzenoneindophenol at 600 mμ exhibits a primary photochemical reaction, for during photosynthesis, water-splitting reduction provides the chemical reducing power which is trapped by the dye, and consequently the dye is reduced to a colorless form. Chloroplastin extracts that showed active dye reduction were observed to have an absorption peak at 488 mμ (one of the Euglena carotenoids) which increased in the light and then decreased in the dark without further addition of dye.[1] This has analogies to the bleaching and regeneration of the visual complex, rhodopsin[18].

Chloroplastins which actively photoreduced dye were also tested for their ability to cause photolysis or evolution of oxygen. Photolysis was measured manometrically in completely anaerobic Warburg Vessels, made oxygen-free to permit a qualitative identification of oxygen with yields of 20 to 30 μl of O_2 in two minutes. This also showed a distinct luminescent glow persisting

for a minute after an anaerobic suspension of Photobacterium phosphoreum was injected into the system in darkness[21].

A light-catalyzed conversion of inorganic phosphate into labile phosphate was also observed over a 1 hr. period in a similar anaerobic system containing 6 co-factors and adenosine monophosphate[21]. The reaction vessels contained 2 ml of chloroplastin with a chlorophyll concentration of about 10^{-5}M, 20 μM of Mg ++30 μM of alpha ketoglutarate, 0.3 μM of riboflavin-5-phosphate, 0.6 μM of menadione (Vitamin K_3) 2μM of ascorbate, 5μg of cytochrome-c, 55 μM of adenosine monophosphate, and 4μg of inorganic phosphate. These experiments were immediately repeated with the addition of glucose and hexokinase, and the glucose-6-phosphate formed was determined by triphospho-pyridine nucleotide reduction at 340 mμ in the presence of glucose-6-phosphate dehydrogenase. In this way, 80 to 90% of the inorganic phosphate that disappeared was accounted for as labile phosphate. The phosphate conversion occurring in dark controls was only 3 to 4% of that found in the light. Whether all of the co-factors play a role in the reaction is as yet unknown. These results, however, do indicate that some typical photosynthetic reactions can be observed with Euglena chloroplastin.

STRUCTURE AND ENERGY TRANSFER

The ordered fine structure for the photoreceptors has led to the idea that they bear a close relationship to a solid state system, exhibiting properties such as electronic energy transfer or electronic charge transfer. Experiments have been carried out with monolayers of chlorophyll, chlorophyll plus β-carotene, or β-carotene alone spread on various surfaces. Such sandwiched models have been demonstrated to be photoconductive[22,23]. Experimental evidences including the electron spin resonance (ESR) experiments and the semiconduction and thermoluminescence studies by Arnold and Clayton[24], suggest that there is a photoconductive mechanism of energy transfer actively participating in the chloroplast. We have found that there is a temperature dependence of conductivity in isolated chloroplasts and chloroplastin similar to the relationship found in semiconductors. These experiments indicate that there is an increase in conductivity to light particularly in the region 660 mμ, also that the conduction is probably by ions, electrons, and holes[25].

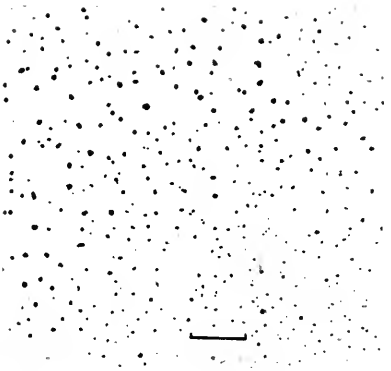
It will be remembered that in order to see the lamellae, the chloroplasts are fixed with osmium tetroxide, potassium dichromate, potassium permanganate, or other metal-containing compounds,

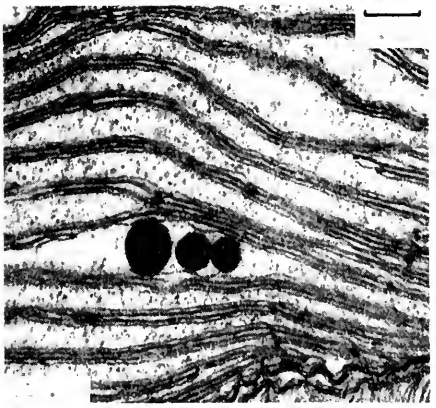
and then dehydrated. This can be considered a kind of Liesegang Phenomenon (in which the fixation is not continuous but forms a series of precipitated concentric rings separated by clear spaces in the protein). Light can modify these periodic structures if the precipitated molecules are light sensitive. If a 2% aqueous digitonin solution (the concentration for extracting the pigment complex) is evaporated from a layer, periodic ring structures will be formed. In addition, digitonin exhibits flow birefringence, and has properties of a liquid crystal. The digitonin micelles have a very strong attraction for the dye molecules, chlorophyll and carotenes. If chloroplastin is allowed to stand, no birefringence is observed; however, a liquid-crystalline phase will separate out, and under certain conditions birefringent rod-like fibers (tactoids) form. These experiments would indicate that the pigment molecules become aligned in the digitonin micelles.

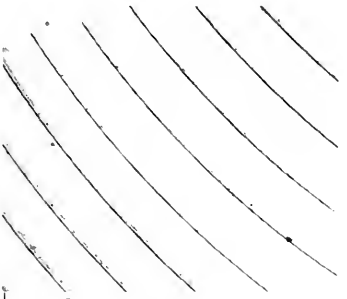
The rapid evaporation of water from chloroplastin will produce periodic ring structures in which the chlorophyll molecules become oriented (Fig. 9a). When these structures are analyzed by scanning the rings and interspaces with the microspectrophotometer at 675 mμ and 550 mμ near the maximum and minimum absorption peaks for chlorophyll in the chloroplast, and the pigments are found in the rings, not in the interspaces (Fig. 9b). Therefore, in the chloroplastin micelles, we not only have chlorophyll, carotenoids, cytochromes, lipids, and protein in the same relative concentration, but in similar orientation as in the chloroplast. The lamellar structure for the orientation of the pigment molecules must be an efficiency mechanism for light capture, but it may also be a critical functioning device.

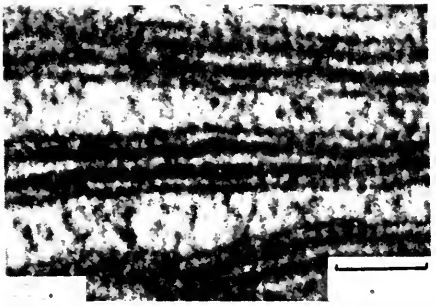
It has been suggested that the role of the detergent micelle in reacting with one of the substrates simultaneously attracting the other substrate to the same vicinity, parallels the behavior of an enzyme in bringing the reactants together; the porphyrin-micelle being analogous to an enzyme-substrate complex[25]. Chloroplastin then has features in common with an enzyme-catalyzed system.

Finally, it is necessary to develop microanalytical methods to study the in vivo chloroplast chemistry and molecular structure. Chloroplastin micelles offer for the present an excellent experimental model system to further pursue the mechanisms of the photoreceptor chemistry to its molecular structure.









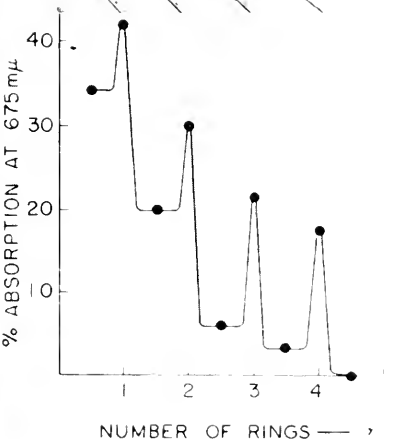


Fig. 5 Euglena EM cross-section showing chloroplasts; Fig. 6 higher magnification of chloroplast section showing the lamellae; Fig. 7 EM showing the globules 50-150 Å in diameter of the chloroplast lamellae; Fig. 8 chloroplastin particles EM, not fixed or stained; Fig. 9a chloroplastin rapidly evaporated from a drop on a glass surface; b scan of the layers at 675 mμ indicating the orientation of chlorophyll in the layers.

Jerome J. Wolken

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VIII. MECHANISM OF PHOSPHORYLATION, AND STRUCTURAL DEFORMATIONS

CONTROL OF CHLOROPLAST STRUCTURE BY ADENOSINE TRIPHOSPHATE

Lester Packer

It is now clear that the membranes of mitochondria (1-2), chloroplasts (3), and photosynthetic bacteria (4) manifest structural changes that are coupled to energy transfer reactions. These changes in membrane structure when measured by the recording of a physical parameter such as light-scattering are found to be closely correlated with conditions favorable for either oxidative or photophosphorylation. Since the reactants required for phosphorylation coupled to electron flow are also necessary for demonstrating structural changes in both photosynthetic and non-photosynthetic systems, and further, since inhibition of these functions abolish the structural changes, it has been suggested that the structural parameter is under the control of dark energy containing intermediates (1-4).

Conditions which affect ATP hydrolysis and synthesis have often been implicated in the control of membrane structure. In mitochondria (5-6) and mitochondrial membrane fragments (7-8), ATP plus a divalent cation such as Mg^{++}, may serve to drive structural changes, which, in the case of intact mitochondria involve shrinkage. Recent experiments with mitochondria (9) have demonstrated that swelling induced by electron transport is oligomycin insensitive, whereas reversal of swelling by ATP (under conditions of oxidative phosphorylation) is specifically blocked by oligomycin. These observations suggest that ATP and its hydrolysis can be involved in the control of mechanochemical changes. Ohnishi and Ohnishi (10) have isolated a protein from mitochondria which undergoes conformational changes with ATP (as indicated by light-scattering and viscosity changes) and manifests ATPase activity. It is therefore an attractive hypothesis that in mitochondria, a contractile-like substance may be involved in the changes in macromolecular structure coupled to energy transduction.

The action of ATP on chloroplast structure has only recently been investigated (11-12). Itoh, Izawa and Shibata (12) using the Coulter counter have discovered that whole spinach chloroplasts shrink reversibly in the light to become 50-80% of their previous volume. The time course of this phenomenon was relatively slow, requiring one to two hours in the dark for reversal of

the shrinkage. A more rapid and reversible type of structural change in chloroplasts has also been reported by measurement of changes in "rectilinear attenuance" (13), which are thought to result from changes in the axial ratio of chloroplasts as shown by a correlated morphological study (12). The addition of ATP to chloroplasts incubated either in the light or in the dark, brought about a shrinkage which was quite rapid; however the circumstances giving rise to this effect appear still somewhat uncertain. In view of this, it seemed worthwhile to examine in more detail the role of ATP in the control of chloroplast structure.

The results of a typical experiment demonstrating structural changes in chloroplasts isolated from spinach leaves is shown in figure 1. Initially, the chloroplasts were incubated under conditions necessary for phosphorylation except for phosphate and actinic light. The reaction system contained phenazine methosulfate (PMS), ADP, and Mg^{++}. A low concentration of chlorophyll was employed to prevent pigments from interfering with light-scattering measurements. The light-scattering responses were measured with a low intensity green light located near the minimum of the photochemical action spectrum since this process minimizes the possibility of activating electron flow by the incident beam. The combination of low chlorophyll concentration and low intensity green light is the trick for demonstrating scattering responses.[1] The experiment shows that in the absence of phosphate a small light scattering increase of approximately 5% was brought about by illumination of the reaction mixture with actinic red light (6×10^{16} quanta/sec in the range 600-700 mμ). The 5% increase in scattering was reversible upon removal of actinic light. Phosphate (5 mM) was then added in the dark; this led to no change in the scattering level, but upon illumination with actinic light, an enormously enhanced scattering was obtained and it now seen that the scattering increase was about 70% above the initial level. It may be further noted that these responses are repeatable; alternating periods of light and darkness continued to give the scattering response.

Similar studies established that light-scattering increases in

1 Control experiments showed that these optical changes were dependent on the angle at which the emitted green light was measured in a manner characteristic of scattering and that they are therefore not fluorescence changes. Absorbance changes were ruled out by showing that the % change was independent of the chloroplast concentration in the range used in the experiments, and also because the absorbance of the pigments was too low to effect an apparent scattering measurement.

chloroplasts occur under conditions of non-cyclic as well as cyclic photophosphorylation. In non-cyclic systems, using either NADP or ferricyanide as electron acceptors, scattering responses induced by actinic light are completely abolished by 10^{-6}M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) which blocks the first light reaction. However the ability to manifest scattering changes can be restored by adding ascorbate and the dye 2,6 dichlorophenol indophenol which reinstitutes electron flow and phosphorylation by passing the DCMU block. Scattering changes under conditions of cyclic or non-cyclic photophosphorylation are abolished by 1 mM NH_4^+ or by one of a number of uncoupling agents such as m-chloro-carbonyl cyanide phenylhydrazone (CCP). The magnitude of the scattering response also varies with the intensity of the actinic light, although a careful study of this relationship for several non-cyclic and cyclic systems which support this phenomenon has not yet been made. In brief, all of the known effects of actinic light, electron carriers, phosphate acceptors, and inhibitor substances in the chloroplast system seem to promote or prevent scattering responses in an exactly predictable manner.

While these studies establish that light-scattering increases in chloroplasts occur under conditions of photophosphorylation, the puzzling observation was made that these structural changes were lost in some cases even when chloroplasts were incubated with the cofactors for photophosphorylation. These "aged" chloroplasts developed a requirement for ATP to manifest scattering changes. Figure 2 demonstrates this ATP requirement. Illumination of chloroplasts under conditions for cyclic photophosphorylation led to an increase in scattering of 2% upon illumination with red light. This small scattering increase was reversed after extinguishing actinic light. The addition of 3.3 mM ATP in the "dark" did not change the scattering level. However, when the red light was restored, a rapid and extensive increase in scattering ensued, reaching a steady state at a level 85% higher than the initial scattering intensity. This increased scattering state could be fully reversed by extinguishing the actinic light. A second light and dark period led to a similar cycle of scattering increase and decrease. The large reversible scattering responses, characteristic of "fresh" chloroplasts, had been restored by ATP. A similar restoration of the response can be obtained with ITP.

This remarkable restoration of the scattering response induced by red light in the presence of ATP suggested that this action of ATP might bear some relation to the existence of a light-induced ATPase in chloroplasts reported by Avron (16) and Petrack and Lipmann (17). Petrack and Lipmann demonstrated that light-induced ATPase of spinach chloroplasts was maximally activated in the

presence of cysteine (0.08 M). Accordingly the action of ATP and also of ITP on the scattering responses in fresh chloroplasts was tested in the presence and absence of cysteine. Parallel determinations were made of nucleoside triphosphatase activity. The results (Table I) confirm the existence of the light activated ATPase observed by Petrack and Lipmann, and also show photohydrolysis of ITP. Under conditions where nucleoside triphosphatase

Table I

CORRELATION OF PHOTOHYDROLYSIS OF ATP AND ITP BY SPINACH CHLOROPLASTS WITH CHANGES IN CHLOROPLAST STRUCTURE

	% scattering change		μmoles phosphate formed/mg chlorophyll/15 min	
Condition	Light	Dark	Light	Dark
ATP	+22	-18	0.37	0.38
ITP	+21	-21	0.10	0.22
Cysteine+ITP	+83	-21	1.11	0.12
Cysteine+ATP	+69	- 9	2.73	0.39
Lipoic + ITP	+70	-69	6.4	0.52
Lipoic + ATP	+75	-20	5.5	0.6

activity is maximum (i.e. in the presence of cysteine), it may be seen that the scattering increases induced by red light under conditions of cyclic photophosphorylation are considerably larger as compared with those in the absence of cysteine. The effects of a second thiol compound, reduced lipoic acid, are also shown; its action on scattering responses and nucleoside triphosphatase activity are comparable to those observed with cysteine. Other experiments verified that lipoic acid is more effective on a concentration basis than cysteine for the activation of scattering and nucleoside triphosphate hydrolysis. The scattering changes in the absence of cysteine or lipoic acid, when the triphosphatase activity is lower, were largely reversed in the dark. However in the presence of cysteine or lipoic acid and ATP, the scattering increases were only slightly reversed when the red light was turned off. This was consistently observed with ATP, but less frequently when ITP or GTP were added.

The nature of this incomplete return of the scattering response occurring in the presence of thiol compounds and ATP upon removal of actinic light, was further examined as illustrated by figure 3. Figure 3 shows that in the presence of cysteine, ATP and basic reaction mixture, chloroplasts manifested an incomplete return of scattering after removal of actinic light. Assuming that this incomplete return was a consequence of light activated hydrolysis of nucleoside triphosphates, it seemed of interest to examine the action of a number of substances which have been reported to inhibit this activity in chloroplasts. NH_4Cl and ADP, added at con-

centrations equal to that of ATP (2 mM) brought about a rapid completion of the reversal of the scattering response in the dark. Abolition of the original scattering increase by NH_4Cl was virtually complete, where as ADP led to only a partial reversal. When the ADP concentration was raised in a stepwise fashion, to twice or three times the concentration of ATP, however, the cumulative reversal was as great as that with NH_4Cl. The kinetics of the reversal of scattering brought about by ADP and NH_4^+, show that the rate of the dark return was more rapid with ADP than with NH_4Cl.

In agreement with the action of ADP on reversal of scattering changes, it is found that this substance inhibits nucleoside triphosphatase activity under the same conditions that it brings about reversal of the scattering level maintained in the presence of ATP. Furthermore, in the absence of Mg^{++} or a thiol compound, reversal of scattering is complete without any additions and under these conditions, the corresponding values for ATPase are low. It is therefore concluded that high ATPase is correlated with incomplete reversal of scattering. The action of NH_4Cl on the other hand, must be different from ADP since NH_4Cl is not an inhibitor of ATPase under these conditions (18-19). The action of NH_4Cl on scattering responses deserves further consideration since it is a powerful inhibitor of photophosphorylation. In the absence of thiol compounds, NH_4Cl added before illumination prevents scattering responses brought about by actinic light; in the presence of a thiol compound however, scattering responses can still proceed. In the presence of cysteine and ATP, for example, scattering increases brought about by actinic light were approximately the same whether NH_4^+ was present or not; with lipoic acid, the scattering increases in the presence of ammonia were 40% of those in its absence. The action of NH_4Cl on scattering and its lack of effect on nucleoside triphosphatase activity, suggest that this substance acts at different site in the dark energy transfer pathway in chloroplasts, to that involved in nucleoside triphosphate hydrolysis.

Since these results suggested an involvement of light activated nucleoside triphosphatase activity in the control of scattering changes geared to photosynthetic electron transport, the action of ATP and the requirement for actinic light were therefore further examined. Recently (18,20) it was discovered that the actinic light requirement for the light activated nucleoside triphosphatase is indirect; the optimum conditions for stimulating this activity were found to be a brief period of pre-illumination in the presence of a thiol compound, a redox factor such

as PMS and Mg^{++} ($t_{1/2}$ for activation = 70 secs). In view of this it was of interest to examine the action of ATP on chloroplast structure in the dark, following brief periods of pre-illumination. As shown in figure 4, pre-illumination of chloroplasts in the presence of the complete system with ATP, even after extremely short light intervals, resulted in a pronounced effect of ATP on chloroplast structure in the dark. Eventually, after 60 secs pre-illumination, the light-scattering first decreases, and then increases in the dark; thus arriving finally at the condition observed after the more prolonged 5 min illumination (cf. figure 3).

To define more precisely the action of ATP, an experimental design was developed to permit the simultaneous measurement of its hydrolysis measured by pH change with light-scattering. Control experiments showed a linear relationship between the extent of pH decline and ATP expenditure. Calculations of the rate of ATP hydrolysis made by the pH method closely corresponded with chemical analysis. In view of this, it was possible to follow the time course of ATP hydrolysis with light-scattering changes. Figure 5 shows the typical light-scattering responses under conditions of ATP hydrolysis. The pH trace indicates the requirement for pre-illumination for activation of ATP hydrolysis. After removal of actinic light, hydrolysis of ATP proceeds in a linear fashion and an incomplete reversal of the scattering response characteristic of these conditions is observed. Upon exhaustion of ATP in the dark as indicated by the cessation of the pH change, a complete reversal of the scattering level to the original dark level is observed. This experiment provides proof that ATP and its hydrolysis are responsible for maintenance of the steady state level of scattering change observed in the dark.

It was therefore of interest to ascertain the action of ATP on the steady state of scattering under conditions favorable for both synthesis and hydrolysis. This was tested under conditions of continuous illumination and it was found that the scattering change was 48% above the dark control. Upon exhaustion of ATP in the light, a decline of the steady state of light scattering change to 38% could be observed.

In view of the close correlation of the action of ATP and its hydrolysis to conditions necessary for generating scattering changes in chloroplasts, it seemed possible that the action of ATP might be associated with the presence of some contractile principle. Accordingly, chloroplasts were extracted under the same conditions employed for the extraction of actomyosin from mammalian muscle. A protein fraction could be extracted from chloroplast membranes which hydrolysed ATP and ITP. The fraction also showed decreases in light-scattering on addition of these nucleo-

tides. Parallel studies on the viscosity of the protein extract made in the presence and absence of the nucleotides did not show a decrease in viscosity with added nucleotides, which would be expected if this preparation behaved like the contractile proteins of mammalian muscle and mitochondria. Also the substrate specificity of the chloroplast protein extract was examined; hydrolysis of ATP, ITP, ADP, PP_i and p-nitrophenyl phosphate occurred indicating a rather non-specific phosphatase activity. These results differ from those for light activated ATPase in whole chloroplasts (18), which are unable to hydrolyze phosphate compounds other than nucleoside triphosphates. Either the extracted protein fraction represents a different nucleoside triphosphatase or non-specific phosphatase, or alternatively extraction of this material from the chloroplasts leads to a change in its properties. In view of these differences, it is difficult to decide on the precise relationship of this extracted material to the action of ATP and ITP in chloroplasts without further study.

DISCUSSION AND CONCLUSIONS

The findings presented here demonstrate that ATP exert a definite effect on the scattering responses of chloroplasts which is related to its light activated hydrolysis. It is also demonstrated that the conditions required for photophosphorylation are capable of promoting scattering changes in chloroplasts in response to actinic light. Thus two possible routes may exist for these effects in chloroplasts. Referring to the diagram illustrated in figure 6, the present results may be explained by analogy with the energy transfer pathway by mitochondria in the following fashion. The photosynthetic electron transfer pathway would lead to the production of a series of intermediates, the earlier non-phosphorylated intermediates eventually becoming phosphorylated, and by interaction with ADP leading to the synthesis of ATP. In view of the results with light activated ATP hydrolysis, including the action of reactants such as ADP and ammonia, the site in the dark energy transfer pathway most closely associated with scattering changes may well be located in the region of the non-phosphorylated intermediates, before the terminal stages where ATP synthesis and hydrolysis would take place. The marked inhibitory effect of ammonia on scattering responses but not on ATP hydrolysis after it has been light-activated supports the notion that this region of the energy transfer pathway, close to the electron transport system, is involved with the scattering changes and that this is the region which is affected by ammonium ions.

Should this analogy between similarity in the dark energy transfer reactions in mitochondria and chloroplasts be further

substantiated, it may be that alternate routes of energy transduction are possible. Thus intermediates in the dark energy transfer pathway may be generated either by photophosphorylation or reversal of the terminal steps by ATP. Both of these conditions lead to favorable circumstances for scattering changes in both mitochondria and chloroplasts. Since these conditions also lead to specific cation accumulation mechanisms in mitochondria, it seems possible that an ion accumulation mechanism driven by these reactions may also exist in photosynthetic membrane systems. This hypothesis, if established, may warrant some evaluation in terms of the quantum efficiency of the photosynthetic energy conversion process.

Finally, no conclusion can, at present be made, concerning the exact correspondence of light-scattering changes under the conditions described here with changes in chloroplast structure. However some correlation between scattering responses and structural changes are supported by the very interesting observation made independently by Itoh, Izawa and Shibata (12) involving entirely different techniques. The observation of Itoh et al. (12) are also substantiated by recent studies made in our own laboratory by means of rapid time recordings of viscosity changes in chloroplast suspensions observed with a rotating viscometer (21). The nature of the physical change of which scattering and viscosity changes may be a measure are currently under active investigation.

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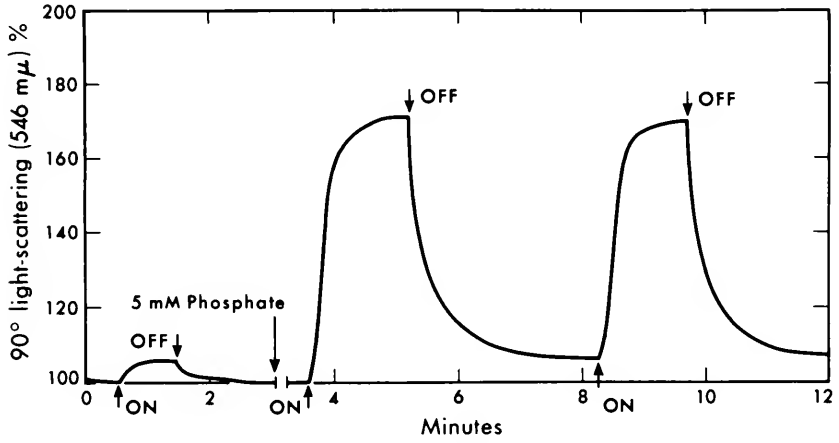


Figure 1. Action of phosphate on scattering changes induced by red light in spinach chloroplasts. The reaction system contained Tris (0.02 M, pH 8.0), NaCl (0.035 M), $MgCl_2$ (0.005 M), ADP (0.001 M), ascorbate (0.001 M), phenazine methosulfate (20 μM), and chloroplasts (5 μg/ml chlorophyll). Temperature was accurately controlled during periods of illumination at 25° ± 0.1°C, by circulating liquid around the jacketed cuvette in the light-scattering photometer. Scattered light was filtered at 546 mμ to prevent interference by actinic red light.

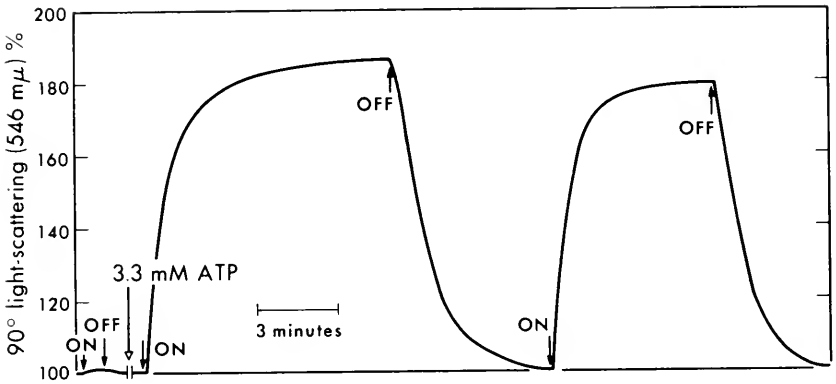


Figure 2. Restoration of reversible scattering changes in aged chloroplasts by ATP.

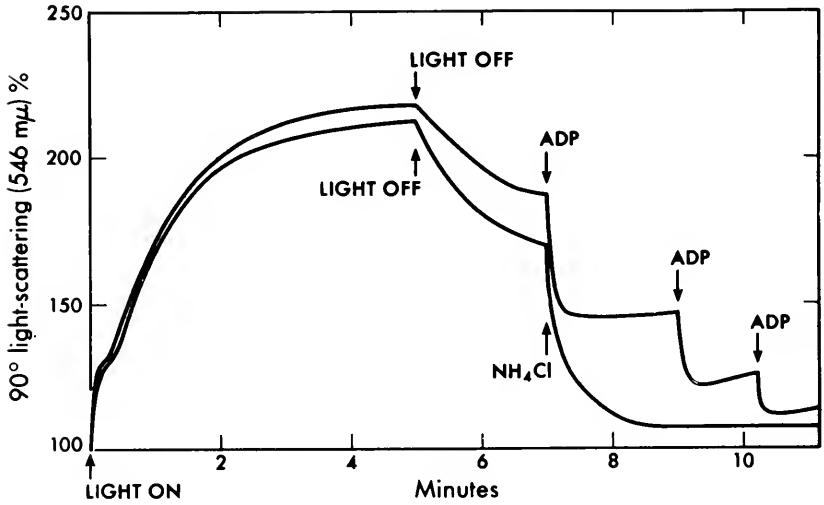


Figure 3. Incomplete reversal of chloroplast scattering under conditions of ATP hydrolysis. Conditions as in figure 1 plus cysteine (0.08 M), and ATP (2 mM). ADP (2 mM) in one experiment and NH_4Cl (2 mM) in the other were added as shown.

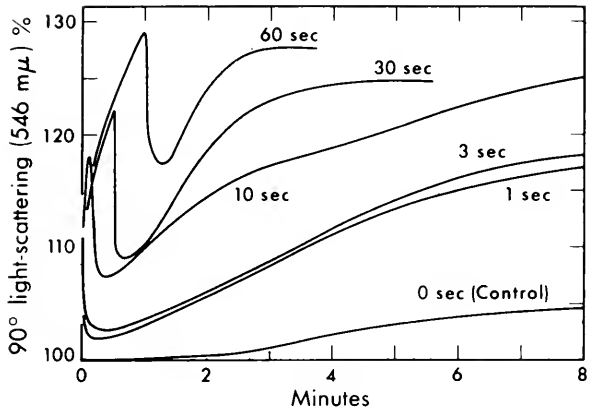


Figure 4. Role of pre-illumination time on dark action of ATP on chloroplast structure. Conditions for scattering experiments as in figure 3. To start the experiment 1 mM ATP was added in the dark, and 60 seconds afterwards actinic light was turned on for the times indicated on the curves.

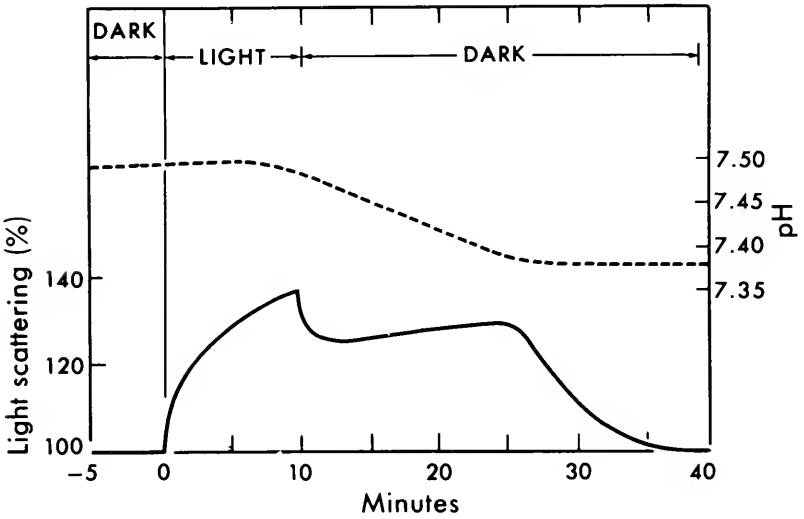


Figure 5. Time course of ATP hydrolysis and scattering changes in chloroplasts.

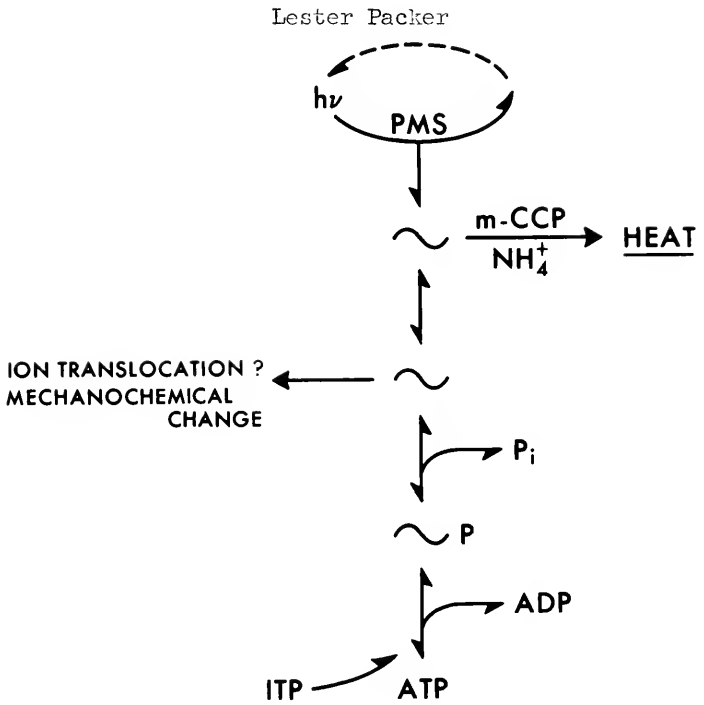


Figure 6. Hypothetical scheme for dark energy-transfer reactions in chloroplasts.

STUDIES ON THE MECHANISM OF PHOTOPHOSPHORYLATION

A. T. Jagendorf and Geoffrey Hind

Our search for insight into the mechanism of ATP formation by chloroplasts has been based, in the recent past, on a two stage technique. The principle is to illuminate chloroplasts together with some, but not all, the components necessary for phosphorylation of ADP; then turn off the light and add the missing compounds. Such a procedure should be capable of providing information on the existence and survival time of intermediates between the initial light absorption, and the final pyrophosphate bond.

The first attempts with this approach were interpreted as showing the existence of a high energy phosphorylated intermediate (1). Results with improved techniques (cutting the time interval between illumination and dark additions to a fraction of a second, by illuminating in a syringe, and injecting directly into a completely dark test tube) now lead us to believe that the earlier interpretation was probably not correct. The earlier results may be explained instead by something unsuspected at that time: the existence of a light-induced transient ATPase which is strongly inhibited by ADP at concentrations as low as 20 uM. This phenomenon is distinct from the two reported ATPases (4,5,6) in certain of its requirements and should be helpful in the elucidation of photophosphorylation from the reverse direction.

The more rapid injection technique has shown that a non-phosphorylated intermediate (or intermediate state) of the chloroplasts does exist, and in major amounts under the proper circumstances. Although it has not been identified, we have found a correlation of the high energy state of the chloroplasts with both a very major pH change, and with a non-specific alteration in the optical density, probably representing a change in chloroplast geometry.

Light activated ATP breakdown.

Table 1 shows the fate of the charcoal adsorbable counts formed by illuminating chloroplasts with P^{32}, and no added ADP. Two features - the loss of counts in the

dark and the partial stabilization by ADP made us hope at first that we were observing a phosphorylated intermediate. However our efforts at identification by chromatography, luciferase assay, etc. showed the counts to be in free ATP^{32} - presumably arising from endogenous ADP. This ATP^{32} is split by the ATPase unless either TCA or a sufficiently inhibitory concentration of ADP is added.

Table 1. Breakdown of newly formed ATP^{32}

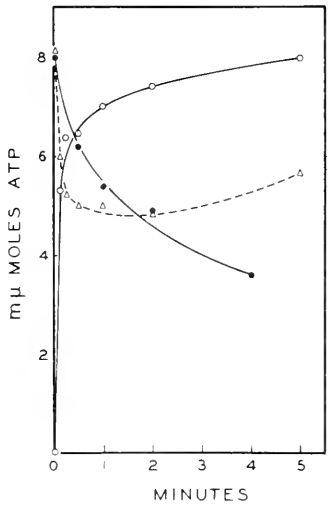
ADP in dark	ATP^{32} Recovered After Dark Incubation (seconds)				
	0	2	17	32	180
—	3.38*	2.45	1.64	1.43	.85
+	—	2.45	1.94	1.85	1.53

*mumoles P32/mg chlorophyll, adsorbed to charcoal. All samples exposed to light in a syringe, for 30 seconds at 22 C and pH 8. Light stage contained once washed chloroplasts with 250 ugm of chlorophyll, Pyo. .05 mM, $MgCl_2$ 5 mM, NaCl 10 mM, NaH_2PO_4 labeled with P-32 0.33 mM, and Tris buffer 17.5 mM; total vol. 1.0 ml. After illumination the samples were kept in the dark for the times indicated, prior to adding to .20 ml of 20% trichloroacetic acid for killing. Injection into ADP in all cases was done after 2 seconds dark in order to allow for decay of X_e. (ADP, 0.5 mM).

These experiments cast doubt on the conclusions of Kahn and Jagendorf (1). In the first place, internal ADP was supposed to have been removed in the earlier work by conversion to cold ATP in a preillumination period. Table I shows that any such transition would have been less than permanent, so ADP probably was present. Secondly, the earlier technique involved handling eight samples at a time, with a consequent lack of precise control over the time of addition of various

reagents. As a matter of fact, TCA-only addition was generally made several seconds later than ADP addition. The shifting control values in Table I show that such a procedure is dangerous. ADP can lead to recovery of more ATP^{32} because it stabilizes some of the light product, rather than because it leads to the formation of new ATP^{32} in a dark reaction. Finally, if ADP is added soon enough it can also pick up the last remnants of the non-phosphorylated intermediate. Although a high energy phosphorylated intermediate may truly exist, the previous experiments can not be considered to have demonstrated it critically. More powerful and direct techniques - perhaps those of Hinkson and Boyer (7) will be needed for a more definitive demonstration.

The ATPase that forms in the light, in our case, has been shown to be particulate and transient (manuscript in preparation). Trace amounts of ATP^{32}, whether formed in the light or added later, can be broken down by properly activated chloroplasts. Activation consists of incubation in the light with a trace of ADP, and pyocyanine. Interestingly, the time course for activation appears to be identical with that for phosphorylation if some phosphate is present as well as ADP. (See Figure 1).



Jagendorf and Hind

Figure 1. Time courses relevant to light induced ATP-ase.

●—● Dark hydrolysis of ATP formed by two minutes' illumination of chloroplasts with labeled phosphate (70 mumoles), $MgCl_2$ (1 mM) and ADP (7.5 mumoles). Δ---Δ Light activation of ATP-ase. Chloroplasts illuminated for duration shown with cold phosphate, magnesium and ADP (4.0 mumoles); then immediately incubated with labeled ATP (3.5 mumoles) for two minutes in darkness. O—O Phosphorylation of ADP accompanying light activation. Chloroplasts illuminated for times shown with labeled phosphate, magnesium and ADP (7.5 mumoles); then injected into TCA. Chlorophyll 250 ug. Pyocyanine 50 uM. Volume 1 ml. pH 8.0 and 5°C.

The ATP breakdown that we see differs from that observed by Avron (5) in that Ca ions added in either the light or the dark stage inhibit the present activity. It differs from the ATPase observed first by Petrack and Lipmann (4,6) in that high concentrations of -SH compounds are not added to accomplish the light activation. It may differ from both of them in greater sensitivity to inhibition by adenylates. Our feeling is that the low ADP concentrations used in these experiments (ca. 5 uM) seems closer to physiological than in the more usual ATPase or photophosphorylation experiments.

Non-phosphorylated intermediate. The very first experiments using chloroplasts illuminated in a syringe produced the startling discovery that the complete phosphorylation reaction, from added ADP, phosphate and magnesium, could occur in the dark. (This result showed up in a control, included as a gesture in the direction of symmetry and completeness, rather than by forethought). Clearly in the light an intermediate was being formed, having very great lability during the subsequent dark period. Its dark decay proceeded with a half-life of 0.5 seconds at pH 8 and room temperature, or about 2 seconds at 3°C (2). We have designated this intermediate as X_e, and define it by the ability to make ATP^{32} in the dark, from added P^{32} and ADP. Very similar results were reported independently by Shen and Shen (8).

In this two stage procedure we are able to discover the effects of varying conditions on each stage separately. One of the most rewarding variables was that of pH (2), since it turned out that much higher yields were obtained on lowering the light stage alone to pH 6. The major effect appeared to be slowing down the dark decay time, to as much as 30 seconds with pyocyanine, or 180 seconds without. ADP, phosphate and magnesium, and the pH 8 optimum, were found to be conditions of the dark phosphorylation steps. As would be expected, calcium (antagonistic with magnesium) and arsenate (competitive with phosphate) inhibit if added to the dark stage alone. Trichlorophenol indophenol dye, recently shown to be an uncoupler of phosphorylation (9,10,11), also inhibits if added only to the dark stage. On the other hand CMU, or uncoupling by removal of cations (12), are effective on the kinetics of the light reaction.

An important point is that if redox cofactors are to have an effect, they must be present in the light stage. Thus X_e must be some entity formed subsequent to, rather than before, electron flow reactions mediated by the redox dyes. In addition, we have ruled out X_e as being a form of pyocyanine itself (2).

The formation, equilibrium level, and dark decay rate of X_e are all affected by the nature of the redox cofactor added (3). Figure 2 shows some kinetic curves for X_e in light and subsequent darkness. The most rapid rate of formation, the highest equilibrium level, and (to a lesser degree) the fastest decay, are induced by pyocyanine. This correlates well with the position of pyocyanine in supporting the fastest rates of one-step phosphorylation. Ferricyanide in some experiments, but not in others, also provides the high equilibrium level of X_e. Other cofactors, including FMN, methyl viologen, PPNR or PPNR plus TPN, usually increase the rate of formation of X_e, but do not lead to equilibrium levels as high as that seen with pyocyanine. Most striking, at pH 6 the lower yield of X_e can be achieved entirely without added cofactors. The rate of formation, however, is quite slow, as much as 3 minutes being required to reach the equilibrium point. It is interesting that the steady state level with pyocyanine is generally twice as high as that found with cofactorless, or with other

cofactors. Although we are occasionally tempted to speculate from this on the existence of two phosphorylation sites with pyocyanine and only one with FMN, etc., in the absence of further evidence this conclusion is questionable.

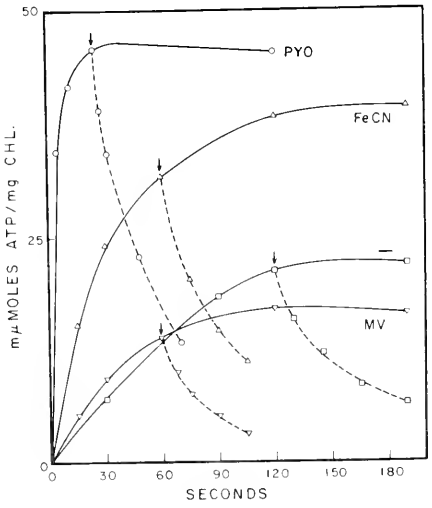


Figure 2. Formation and decay of X_e with various cofactors.

Solid lines: ATP recovered as function of illumination time at pH 6 and 5°C, in white light of 8600 ft. candles. Dashed lines: ATP recovery after turning off light at arrow and giving increasing dark interval before injection into phosphorylating reagents at pH 8.0. Ferricyanide 3.0 mM. Pyocyanine and methyl viologen, 50 uM.

Both the formation of X_e in the light, and its decay in the dark show apparent first order reaction kinetics. The meaning of the formation rate constants seems ambiguous, due to lack of information as to the existence of simultaneous decay processes, or of the total concentration of the reacting species. The kinetic

curves show directly and accurately, however, the rate law for the dark decay reaction, and the steady state level of X_e. The amount of X_e has interest in our attempts to speculate on its nature. With pyocyanine, at pH 6, equilibrium levels have been reported as high as 60 mumoles per mg chlorophyll (2). On increasing the phosphate level, to 10 mM, yields as high as 116 umoles per mg chlorophyll have recently been obtained; and extrapolation of a double reciprocal plot showed a maximum yield of 120 umoles/mg chlorophyll at infinite phosphate concentration. This is a ratio of 1 ATP formed for every 9 chlorophyll molecules. Obviously X_e cannot be a complex of an electron carrier such as cytochrome f, with a coupling factor, as is the case for one or more of the mitochondrial high energy intermediates (10). X_e might involve plastoquinone A, however.

Correlation with absorbancy and pH changes.

With such a major entity any change in absorption spectrum due to the transition to the energetic state should be simple to observe spectrophotometrically. With the kind cooperation of Drs. Britton Chance and Walter Bonner we have attempted to look for such changes. Our criteria for a preliminary correlation with the non-phosphorylated intermediate included dependence on pH 6, and more rapid kinetics and greater amounts with pyocyanine than without. Essentially, we could find no specific absorbancy change in the region between 480 and 590 mu which satisfied the above criteria. Also at 255 mu, in the region of the plastoquinone absorption, we found only a minute spectral change, not dependent on the more acid pH. Thus we are tempted to consider X_e as being related only indirectly to the currently known redox catalysts bound in the chloroplast membranes (with the reservation, of course, that negative evidence is never as conclusive as positive evidence).

However we did observe very large changes in absorbancy at almost all wavelengths examined, amounting to as much as .05 OD unit, with 25 ugm of chlorophyll in a 3 ml cuvette. These changes took as long as 15 or 30 seconds to saturate when pyocyanine was the cofactor, and 2 to 3 minutes without a cofactor. Also, they were pH 6 dependent. In a double beam spectrophotometer deflections were seen even comparing one wavelength

(540 mu) with itself, so light scattering artefacts were implicated. On a split beam instrument it was apparent that actinic light caused an increase in optical density of the suspension. This can even be seen in a Carey spectrophotometer; representative tracings are shown in Fig. 3.

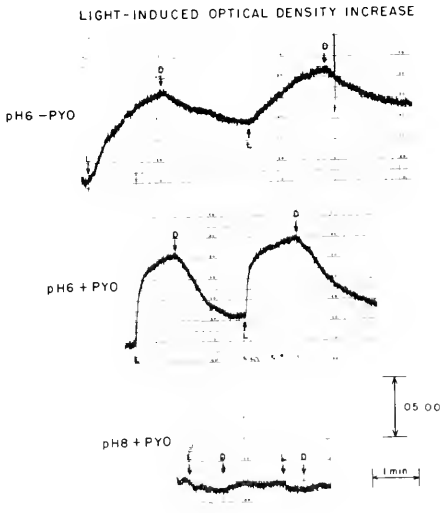


Figure 3. Optical density at 540 mu as a function of actinic illumination with red light (660 mu and up). 25 ugm of chlorophyll in a total volume of 3 ml, containing NaCl 10 mM, 3,3-dimethylglutaric acid and Tris at 20 mM, pH 6.0 or 8.0 as shown. Pyocyanine at .05 mM. Temperature approximately 5°C, measurements in Carey model 14 with simultaneous illumination from above, using microscope illumination lamp filtered through Corning heat filter and Kodak Wratten Gelatin filter no. 70.

The changes is very non-specific for wavelength, occurring from the ultraviolet to 730 mu, at least. In addition the extent of the change is minimized upon using opal glass cuvettes, and maximized by using additional narrow slits before the photomultiplier (14). All of these

things show that some change in geometry or refractive index is occurring. Since preliminary experiments using a Coulter counter, have failed to show any change in total volume, the alteration might be one in axial ratio (15) or refractivity. Since the conditions are so different, it is difficult to say yet whether these effects are similar to the light scattering increments observed by Packer (16) using chloroplasts in a complete phosphorylation mixture.

Our final observation came when we stopped to think about a rather unusual theory. Peter Mitchell (17) has advanced the idea that electron flow, in phosphorylating organelles such as mitochondria and chloroplasts, may lead to accumulation of hydrogen ions inside the prerequisite double membranes. Mitchell calculates that a differential of 3 pH units from the outside to the inside has enough potential energy to accomplish the phosphorylation of ADP, given the appropriate enzymes. We reasoned that if this potential could be generated in the absence of phosphorylation it in itself might account for the phenomena we have labelled "X." Furthermore one consequence of its appearance might be a rise in pH of the medium as hydrogen ions are taken up, or hydroxyl ions secreted, by the particles. Figure 4 shows that when we looked at chloroplasts in an unbuffered medium brought to pH 6 we did indeed see the predicted change in pH of the medium.

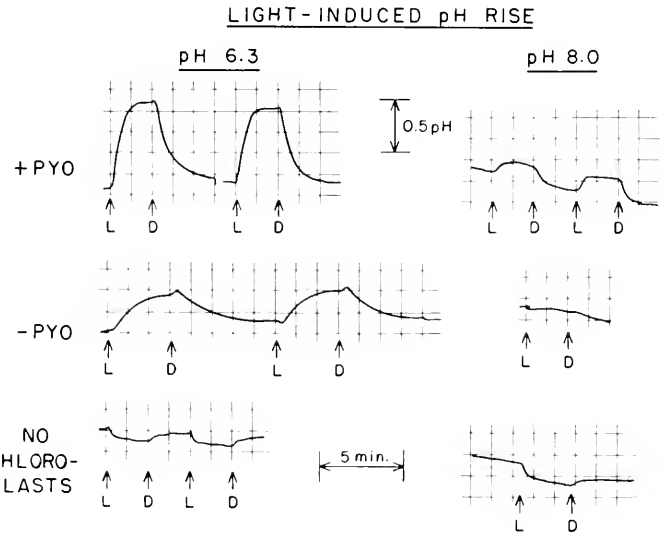


Figure 4. pH shift induced by actinic illumination. Chloroplasts were resuspended in .01 m NaCl and washed once. 2 mg of chlorophyll were used in total vol. of 10 ml, of 10 mM NaCl in a water-jacketed cell at 0°C. Pyocyanine .05 mM when used. Note the electrode response to light, opposite in direction to the pH shift caused by the chloroplasts. Reaction mixture was at pH 6.3, without addition of buffer.

This rise in pH in the light is readily reversible in the dark; is much greater at pH 6 than at pH 8 and is both larger and very much faster when pyocyanine is present than when it is absent. Thus far it correlates quite well with both the kinetics of the intermediate X_e, and the non-specific changes in optical density.

By using a pH-stat to titrate the chloroplasts with acid, the proton uptake (or hydroxyl excretion) can be quantitated. The amount of acid was found to be a linear function of the amount of chlorophyll present, and a catalytic function of the pyocyanine added. The actual numbers have gone as high as 700 muequivalents of H^+/mg chlorophyll, or quite a bit in excess of what one would expect for stoichiometry with X_e (maximal yield 120 umoles/mg chlorophyll). As a matter of fact, the H^+ consumed is practically equal to the total chlorophyll a present. This much of a proton shift is probably consistent with the Mitchell hypothesis as to the role of proton transport in the phosphorylation mechanism. The trouble is, of course, that a pH shift is highly non-specific. It could accompany either ion transport into or out of an internal reservoir, or an actual chemical change. Even if it represents ion movement, the ion transport need not be the cause of the energetic state of the chloroplasts. As in mitochondria (18-21) it might easily be a reaction made possible by the consumption of a small part of the energetic intermediate. A pH shift also accompanies innumerable chemical changes. It seems suggestive, for instance, that transformation of bound histidine to its high energy state as proposed recently by Boyer (22) involves production of hydroxyl ions.

Our current uncertainties include the question of the real function of X_e. We have no way of deciding

yet whether it is an obligate intermediate in the usual process of ATP formation, or some side pool of extra energy. If the latter were the case (and this is suggested but not proven by the large amount and rather slow rate of formation) perhaps its true significance lies in ion transport, or reverse electron flow, or even more directly in CO_2 fixation itself. Indeed, perhaps it is a reservoir of energy that can be used in any of several different ways depending on the circumstances. In the larger view of photosynthesis, an energy reservoir of major proportions in which each unit is of the order of 9,000 calories per mole could be a method of achieving a more flexible use of the total energy of 45,000 calories available from each light quantum.

No matter what the precise relations are between phosphorylation and our three phenomena, it seems fairly safe to guess even now that the ion transport and structural changes in the chloroplast membranes are likely to be intimately linked to the existence and function of high energy intermediates, either on the pathway of or in equilibrium with photophosphorylation. The direction of our future research must be towards finding out the degree of intimacy of these relationships, and which if any of the three is causal for the others.

ACKNOWLEDGEMENTS

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ON THE COUPLING OF PHOTOPHOSPHORYLATION TO ELECTRON TRANSPORT

Mordhay Avron and Noun Shavit

Introduction

The large increase in the rate of electron transport brought about by the addition of the components necessary for phosphorylation was first demonstrated in 1958 with ferricyanide as an electron acceptor[1,2]. It has since been extended to include the other common electron acceptors in photophosphorylation, NADP[3] and indophenol dyes[4], and studied in detail[5,6,7]. A similar and normally somewhat larger increase in the rate of electron flow can also be brought about by uncoupling the chloroplasts from the control of the accompanying phosphorylation. This type of effect results in a concomitant large increase in the rate of electron transport and large decrease in the rate of ATP production. The first demonstration of uncoupling in chloroplasts was provided by Krogmann and Jagendorf[8]. Irreversibly uncoupled chloroplasts were obtained by diluting chloroplasts at pH 6 in the presence of a high concentration of salts. The first demonstration of an uncoupling agent soon followed when ammonium salts were shown to reversibly uncouple photophosphorylation at a concentration around 10^{-3} M [9]. Good has further studied the phenomenon and concluded that a number of monofunctional amines and several anions at high concentrations, can act as uncouplers[10,11].

Figure 1 illustrates the typical response of the reduction of ferricyanide and its accompanying phosphorylation to the addition of the components necessary for phosphorylation,

Fig. 1. Uncoupling of the ferricyanide system by methylammonium chloride. Reaction conditions and assays as previously described[25, 26]. Light intensity, 160,000 lux; reaction time, 2 minutes; gas phase, air; temperature, 20°C; once washed chloroplasts containing 10-20 micrograms of chlorophyll per ml.

or of an uncoupler. It can be seen that the addition of the phosphorylation-components

approximately doubles the rate of ferricyanide reduction. A further addition of an uncoupler increases the rate to an even higher value. While bringing about this further increase in the rate of electron flow the rate of the accompanying phosphorylation drops to zero. Thus, the P/2e$^-$ ratio of about 1 obtained in the absence of the uncoupler gradually decreases to zero as the concentration of the uncoupler is increased. The low rate of ferricyanide reduction, obtained in the absence of the phasphorylation-components is increased by the uncoupler to the same maximal rate obtained in the system containing the phosphorylation-components. This results in a tripling of the rate of electron flow. Increasing the concentration of the uncoupler beyond that required for maximal rate results in a sharp decrease of the rate of reduction.

Potent new uncouplers of photophosphorylation

Until recently ammonium salts were the most potent uncouplers of photophosphorylation known. Attempts to find uncoupling activity in the chloroplast system with the commom potent uncouplers of oxidative phosphorylation have resultedin failure. Thus, dinitrophenol[9] pentachlorophenol[9], and valinomycin[12], have no effect at the concentration in which they completely uncouple oxidative phosphorylation.

Recently several more potent and unrelated uncouplers of photophosphorylation have been discovered. Figure 2 describes the uncoupling effects of atebrin (quinacrine, atabrine) and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (p-CF_3O-CCP). Atebrin is a rather poor uncoupler of oxidative phosphorylation[13]. It was reported by Baltscheffsky[14] to inhibit photaphosphorylation between 10^{-5} - 10^{-6} M. Also, Arnon[15] mentioned that atebrin is an uncoupler of photophosphorylation. As seen in Fig. 2 atebrin acted as a classical uncoupler. 50% inhibition of photophosphorylation was attained at about $1x10^{-5}$ M.

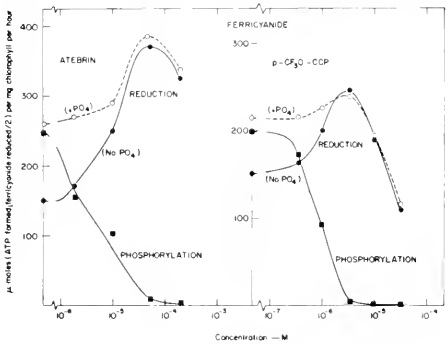

Fig. 2. Uncoupling of the ferricyanide system by atebrin or carbonyl cyanide p-trifluoromethoxyphenylhydrazone (p-CF_3O-CCP). Details as described under Fig. 1.

Several derivatives of carbonyl cyanide phenylhydrazone were shown by Heytler[16] to be potent uncouplers of oxidative phosphorylation and inhibitors of photophosphorylation with phenazine methosulfate as cofactor. Bamberger et al.[17] has recently demonstrated their uncoupling effect on photophosphorylation coupled to NADP reduction. Fig. 2 illustrates the uncoupling effect of the most potent of the derivatives described by Heytler, carbonyl cyanide p-trifluoromethoxyphenylhydrazone, on ferricyanide reduction and the accompanying phosphorylation. 50% inhibition of photophosphorylation was attained at about $1x10^{-6}$ M. Thus, p-CF_3O-CCP is the most potent uncoupler of photophosphorylation

described to-date. The CCP derivatives are also the only group which uncouple both oxidative phosphorylation and photophosphorylation at similar very low concentrations.

Chlorpromazine has been shown by Low[18] to inhibit both electron flow and phosphorylation in oxidative phosphorylation, and by Wessels and Baltscheffsky to inhibit photophosphorylation[19]. Figure 3 shows that it also exhibited all the usual effects of an uncoupler. Pretreating chloroplasts in ethylenediaminetetraacetate (EDTA) in the absence of salts has recently been found in our laboratory to uncouple them (Fig. 3) by releasing from them a coupling factor[20]. Under appropriate conditions this coupling factor can be recombined with the uncoupled chloroplasts resulting in a partial restoration of coupled phosphorylation.

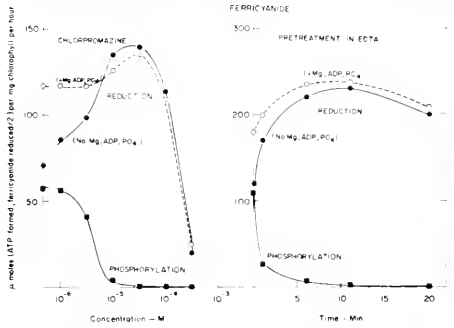

Fig. 3. Uncoupling of the ferricyanide system by chlorpromazine, or by pretreatment of the chloroplasts with ethylenediaminetetraacetate (EDTA). Details as in Fig. 1; pretreatment in EDTA consisted of suspending chloroplast fragments for the time indicated in 1×10^{-3} M EDTA followed by an immediate assay in the various photoreactions.

Differential effects of uncouplers on several photoreactions

One unusual property of some of the uncoupling agents described is shown in Fig. 4. In this figure we compare the effect of three uncouplers on two types of photophosphorylation: that dependent upon the presence of phenazine methosulfate (PMS) and that

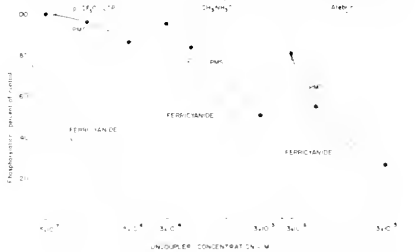

Fig. 4. Differential effect of several uncouplers on phenazine methosulfate (PMS) and ferricyanide dependent photophosphorylations. Details as described under Fig. 1.

coupled to the reduction of ferricyanide. It is evident that whereas atebrin and methylammonium chloride inhibited both types of phosphorylation to a similar degree, p-CF_3O-CCP was a much more potent inhibitor of the phosphorylation associated with ferricyanide reduction than of that dependent upon phenazine methosulfate. The simplest interpretation of such data seems to us to invoke two different sites for ATP production. One associated with the phenazine methosulfate dependent photophosphorylation, and another associated with that coupled to ferricyanide reduction. It may also be recalled that p-CF_3O-CCP has been previously noted to be the only known uncoupler of photophosphorylation which also uncouples oxidative phosphorylation at similar concentrations. It is therefore likely that it acts at a site which differs from the site of action of the other uncoupling agents.

To substantiate such a conclusion it was thought important to repeat these observations under conditions where the large difference in rate between the phenazine methosulfate and ferricyanide dependent phosphorylations was not so apparent. This was achieved by lowering the light intensity until it became the limiting factor in the rate of photophosphorylation.

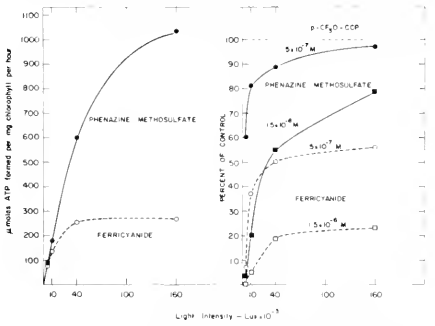

Fig. 5. The effect of light intensity on the rate of ferricyanide and phenazine methosulfate dependent phosphorylations and their inhibition by carbonyl cyanide p-trifluoromethoxyphenylhydrazone (p-CF_3O-CCP). Details as described under Fig. 1.

The left side of Fig. 5 illustrates the response of both types of photophosphorylation to different light intensities. It is evident that whereas the rate of phenazine methosulfate dependent phosphorylation was about four fold higher than that of the one coupled to ferricyanide reduction at 160,000 lux, the two rates were essentially equal at 4,000 lux. The extent of inhibition of both systems by p-CF_3O-CCP at different light intensities is plotted on the right side of Fig. 5. It is evident that the relative inhibition of the two systems does not vary significantly as one lowers the light intensity, and so equalizes the rates of photophosphorylation in the two systems. This strengthens in our opinions, the suggetion that two different sites of photophosphorylation are present in the two systems.

The relation of the extent of uncoupling and inhibition to light intensity

A curious, and as yet uninterpreted phenomenon, which became evident as these studies progressed was the much stronger inhibition of photophosphorylation by the uncoupler as the light intensity was lowered (Fig. 5, right). Fig. 6 illustrates that this effect was found not only with p-CF_3O-CCP, but with all the uncouplers tried. It is also very strongly evident in the case of the inhibition by 3-(3,4, dichlorophenyl)-1,1,-dimethylurea (DCMU). The

latter observation may explain some of the variance in the results of different workers, using markedly different light intensities, as to the inhibition by CMU derivatives on different

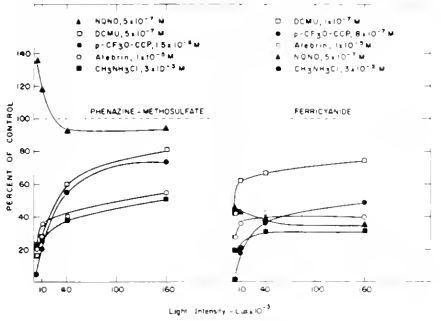

Fig. 6. The effect of light intensity on the extent of inhibition of phenazine methosulfate or ferricyanide dependent photophosphorylations by several uncouplers or inhibitors.
Details as described under Fig. 1.

photoreactions of chloroplasts (see for example, reference 21). The only exception found was the inhibition produced by 2-nonyl-4-hydroxyquinoline N-oxide (NQNO), which decreased, rather than increased, as the intensity was lowered. This is the only basic difference found to-date between the inhibitory effect of this compound and that of the CMU derivatives on the photoreactions of chloroplasts(22).

The dependence of the rate of electron transport on the osmotic concentration

Another effect which leads to a large variation in the rate of electron transport in the various photoreactions of chloroplasts was recently discovered in our laboratory. Figure 7 describes the effect of lowering the salt concentration normally used in the reaction mixture. It is evident that the rate of electron flow in all of the photoreactions illustrated was markedly decreased as the salt concentration decreased. This effect was not due to an uncoupling, since within the concentration used, the salt had no depressive effect on photophosphorylation. Table 1 shows that this was a general osmotic effect with NaCl, KNO_3, or sucrose having a similar effect at the same osmotic concentration. Maximal increase in rate by about twenty fold have often been observed. The table also indicates that with the osmotic concentration employed by most workers, only a slight further stimulation of rate can be achieved by an additional increase in the osmotic concentration.

Figure 8 shows that the extent of stimulation in the rate of reduction of ferricyanide is also affected by the osmotic concentration of the reaction mixture. Thus whereas at low osmotic concentration approximately a five fold stimulation was produced by the addition of the phosphorylation-components or of an uncoupler, less than two fold stimulation was produced at higher osmotic concentration.

The osmotic effect described is reminiscent of the earlier observations on the effect of salts on the rate of the Hill reaction which were performed in attempt to prove or disprove the function of chloride ions as a coenzyme of the Hill reaction(23, 24). It does not seem

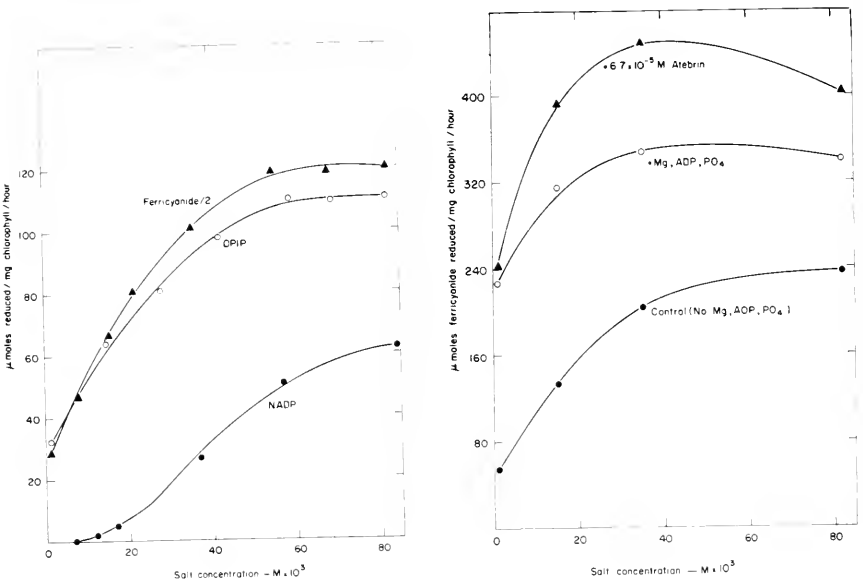

Fig. 7. The effect of increasing salt concentration on the rate of several photoreductions.
Details as described under Fig. 1, except that the reaction mixture contained only 13 μmoles of sucrose per ml, and 1 μmole per ml of TRIS-HCl buffer at pH 7.8. The pH of all reaction mixtures was checked at the termination of the experiments, and was found to be between 7.5 - 7.8. The salt employed was either TRIS-HCl at pH 7.8 or NaCl.

Fig. 8. The effect of salt concentration on the stimulation of the rate of electron transport produced by the addition of phosphorylation-components or an uncoupler.
Details as described under Fig. 7.

to be related to the uncoupling effect of anions, recently described by Good[11], since the latter effect occurred only at higher concentrations than the osmotic effect presently described.

Summary

To summarize, we have indicated several properties related to the coupling of photophosphorylation to electron transport in isolated chloroplasts from swiss-chard leaves. The effect of several new and more potent uncouplers was described. Their differential effect on

Table 1
The effect of osmotic concentration on the rate of photoreduction of ferricyanide

Reaction components	Specific activity	Relative activity
µmoles/ml	µmoles reduced/ mg. chl./hour.	
TRIS (1)	12	1
TRIS (1) + NaCl (20)	166	14
TRIS (1) + NaCl (40)	206	17
TRIS (1) + NaCl (67)	225	19
TRIS (1) + Sucrose (40)	168	14
TRIS (1) + Sucrose (80)	199	17
TRIS (1) + Sucrose (13)	58	5
TRIS (1) + Sucrose (13) + NaCl (20)	182	15
TRIS (1) + Sucrose (13) + KNO_3 (20)	142	12
TRIS (15)	130	11
TRIS (15) + Sucrose (13)	145	12
TRIS (15) + Sucrose (13) + NaCl (20)	199	17
TRIS (15) + Sucrose (13) + NaCl (67)	236	20

Details as described under Fig. 1, except that the only reaction components present, other than chloroplasts ore indicated in the table. The numbers in parantheses refer to concentration in µmoles per ml.

several photoreactions of chloroplasts was interpreted as pointing to two separate sites of ATP production in chloroplasts. The large changes in the extent of inhibition of several photoreactions by uncouplers and other inhibitors as the light intensity was changed was illustrated. Finally, an osmotic concentration effect was described which largely determines the rate of electron flow in several photoreactions.

Acknowledgements

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THE STOICHIOMETRY OF PHOTOPHOSPHORYLATION

Thomas Punnett

When non-cyclic photophosphorylation was first described by Arnon et al (1), the ratio of ATP formed to electrons transported was found to be one. This determination was repeated by Avron and Jagendorf (2), by Davenport (3), by Good (4), by Stiller and Vennesland (5), by Turner et al (6) and others. In all cases, the ATP/2e- ratio has been the same.

There is some ambiguity in this determination, however, because of the nature of the data (2). In a typical experiment, one obtains the rate of phosphorylation, the rate of oxidant reduction in the presence of phosphate acceptor and the rate of reduction in the absence of acceptor. The ATP/2e- ratio can be calculated either by dividing the rate of phosphorylation by the maximum rate of reduction, or by "correcting" the ADP-enhanced reduction rate for the reduction occurring in the absence of ADP and dividing this "corrected" value into the rate of phosphorylation. The usual effect of making this correction is to increase the ATP/2e- ratio from one to two or three. Arnon and Avron and Jagendorf elected to use the more conservative uncorrected value of one while Good has argued in favor of the corrected value of two. Stiller and Vennesland, in their earlier study only,found a good stoichiometric relation but concluded that this relation was fortuitous and that the two processes were not related to each other in a quantitative manner. We have held both of these latter two views at various stages of this investigation.

The first problem, therefore, was that of the treatment of the date. Our approach to this problem was to study reaction rates as a function of several variables to see whether a constant stoichiometry could be obtained. The basic assumption was that if phosphorylation and reduction were the consequence of a single process, then the overall rates should vary with pH, concentration of oxidant, etc., but the stoichiometry should be constant. Furthermore, a constant stoichiometry should be found only with one of the two methods of treating the data.

FERRICYANIDE PHOSPHORYLATION

In these experiments, done with the collaboration of Dr. Rajul V. Iyer, we used oat chloroplasts (Avena sativa, var. Garry) at a concentration of 7 to 20 micrograms $\overset{CHLL}{\wedge}$ per ml., high intensity red light and one to two minute reaction times. In the determinations of pH curves for the ferricyanide Hill reaction, we found that the addition of ADP had no effect on the reduction rates between pH 6.8 and 7.5 although there was an appreciable rate of phosphorylation. When these data were used to calculate an ATP/2e- ratio, the "corrected" ratio was infinite. It decreased to 2 only when the pH was increased to 7.9 and was variable with further increase to pH 8.7. This response, and that described below in the quinone Hill reaction, lead us to conclude that it is improper to "correct" the reduction rates.

If this conclusion is correct, the next question is whether one obtains constant stoichiometry by using ATP/2e- (total) and what is the value of this ratio. The value we have found, 0.70 to 0.85, is in agreement with work published earlier. We have also found in these studies that the uncorrected ATP/2e- ratio was relatively constant over a wide range of conditions. In the ferricyanide Hill reaction, the ratio increased slightly from pH 6.8 to 7.6 and was constant to pH 8.7 (Fig. 1).

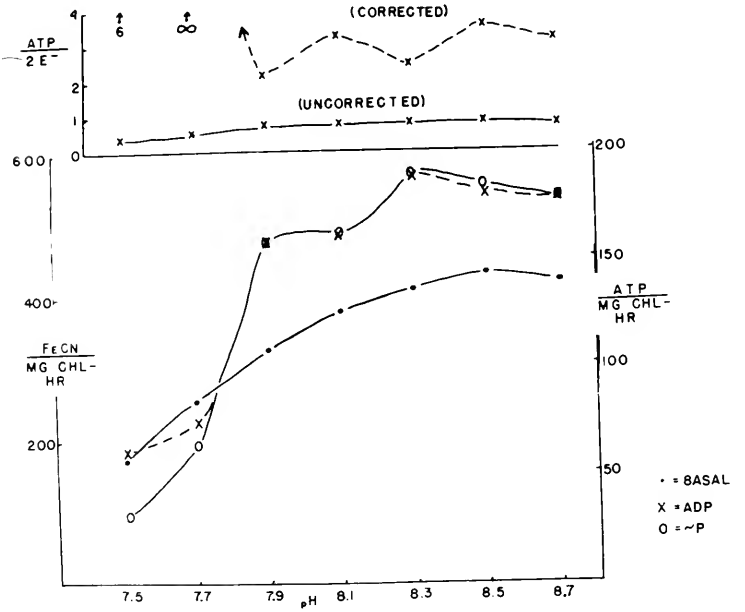

Fig. 1- Reduction, phosphorylation and ATP/2e- ratio accompanying the ferricyanide Hill reaction.

At pH 8.0 the ratio was constant when the ferricyanide concentration was varied from 20 μM/L to 2.2 mM/L. (Fig. 2). The ratio was constant over a wide range of light intensities (done in conjunction with Dr. T.T. Bannister), though it dropped sharply at low intensity as reported by Shen and Shen (7), and by Turner et al (6).

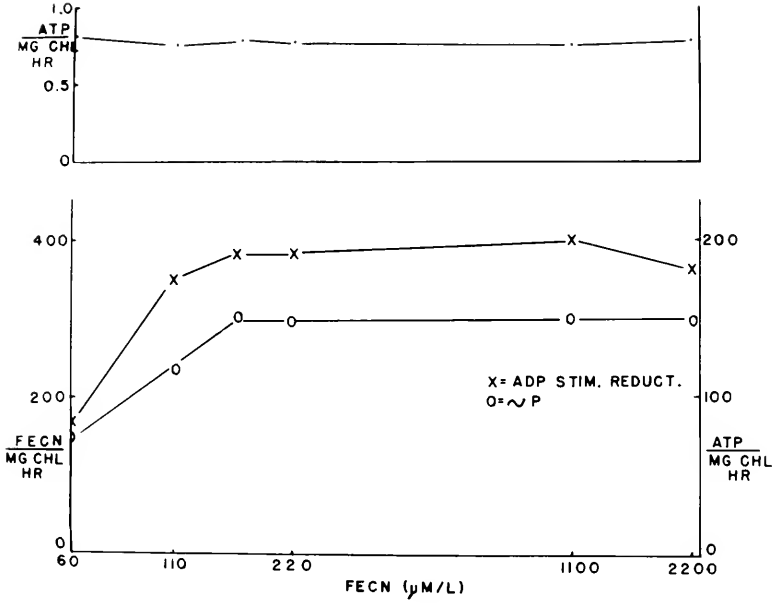

Fig. 2- ATP/2e- ratio as a function of the ferricyanide concentration.

P-QUINONE PHOSPHORYLATION

Another finding was that the quinone Hill reaction is accompanied by a noncyclic phosphorylation. It was necessary to use a low concentration of oxidant (0.11 mM/L quinone) as was the case in Avron's (8) and in Kiester's (9) studies of dye-induced photophosphorylation. One unusual feature of this reaction was that the rate of quinone reduction was not affected by the addition of ADP at any pH. In this case, too, the "corrected" ATP/2e- ratios would have been infinite. The ATP/2e- (total) varied from 0.3 to 0.7 over the pH range 6.8 to 8.4, with the maximum ratio occurring at pH 7.6 to 8.0. (Fig. 3). This ratio was neither as high nor as constant as that obtained in the ferricyanide reaction, probably because of the instability of p-quinone. The quinone ATP/2e- ratio was fairly constant from 5 μM/L to 110 μM/L, while at higher concentrations uncoupling became progressively greater.

With both the quinone and ferricyanide Hill reactions, care must be taken to add CO_2 to the reaction mixture if the oats are grown during the winter. The added CO_2 increased the reaction rates and the ATP/2e- ratios apparantly because it reacted directly with the phosphorylating step.

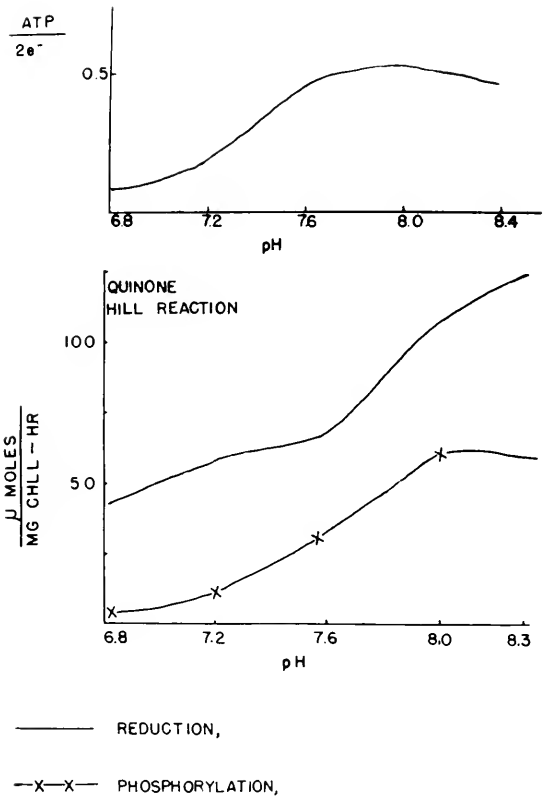

Fig. 3- Reduction, phosphorylation and ATP/2e- ratio accompanying the aerobic p-quinone Hill reaction.

OTHER PHOSPHORYLATIONS

Similar stoichiometries have been reported by Arnon (1), by Avron and Jagendorf (2), by Davenport (3) and by Black et al (10) for the NADP Hill reaction, and by Avron (8) and by Kiester (9) for the DCPIP Hill reaction.

CYCLIC PHOSPHORYLATIONS

In the case of the different cyclic photophosphorylations, Yin et al (11) have reported that the quantum yields for cyclic and non-cyclic ATP production are the same. George Hoch and I have confirmed these results on the relative quantum yields for the phosphorylations induced by pyocyanine, FMN, menadione, NADP and ferricyanide (Fig. 4). The simplest interpretation of these results is that the same number of phosphorylating sites is functioning in cyclic and non-cyclic electron transport.

In summary, the accumulated evidence supports the view that there is one phosphorylating site in the Hill reaction system. A second conclusion supported by these data is that the practice of correcting the reduction rates for the "control" rates clearly leads to values of the ATP/2e- ratio that are unacceptably high. It may be proper to correct the reduction rate for the rate found in the absence of all three phosphorylation reagents, ADP, Mg^{++} and $PO_4^{=}$ rather than in the absence of ADP (12). If so, the correction would be much smaller and would probably have the effect of increasing the ATP/2e- ratio from 0.80 to 1.0. In either case, this ratio still approaches one as a limit.

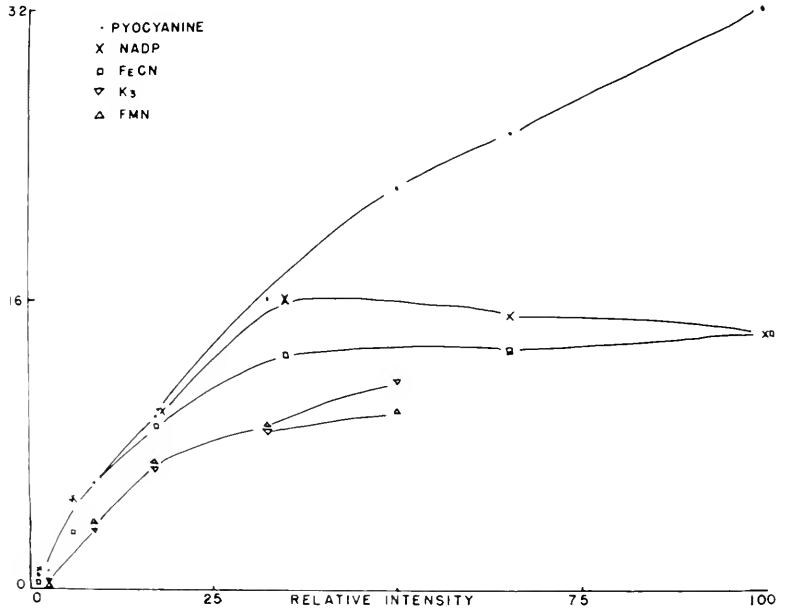

Fig. 4- Relative rates of cyclic and non-cyclic phosphorylation as a function of light intensity.

There may be two or more phosphorylating sites in the electron transport system of the chloroplast, but they are still hypothetical at the moment. Up to the present time, the experimental evidence given in support of the two site hypothesis can be explained in other ways. If a second phosphorylation site exists in chloroplasts, a different kind of experiment will be needed to demonstrate its presence convincingly.

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STRUCTURE-FUNCTION RELATIONSHIPS IN PROTEINS AND THEIR POSSIBLE BEARING ON THE PHOTOSYNTHETIC PROCESS

Rufus Lumry

Structure-function relationships in muscle have been studied for many years. Aside from these, however, such relationships of biological systems at the single molecule level have aroused little interest until the last few years. What little work there is to date is largely confined to single proteins and has attempted to explain the unusual characteristics of the unique physiological reactions of these proteins in terms of conformational factors. A good example is the attempt in the field of vision to show that light produces a conformation change in rhodopsin which converts it from a noncatalytic to a catalytic protein, thus to produce by chemical amplification a sufficient change in chemical composition near an end plate to initiate neurone function.[1] In organized systems such as the mitochondrian there has been the unexplained but highly dramatic occurrence of a swelling and shrinking associated with the integrity of the oxidative phosphorylation system and the rate of phosphorylation itself.[2, 3] In addition, there has been some evidence that physical properties such as strength of adherence of sub-proteins in the mitochondrian depend on specific binding of reagents known to influence in a marked degree the phosphorylation process.[4] According to a recent report of Weinbach, Shefield and Garbus[2] the swelling of mitochondria disappears on removal of the uncoupling reagent. This removal is effected by adding serum albumen which has a higher affinity for the uncoupling agent. ATP must also be added to the suspension. The swelling and shrinking reaction is complex, but seems to involve the muscle-like action of contractile proteins in the mitochondrial membranes. Somewhat similar changes in morphology occur as the steady-state rate of phosphorylation is altered in normal mitochondria. The implications of such findings appear to be that the effects are due to specific chemical interactions and not to minor modifications in osmotic balance; and that there is some connection between the morphological state of the mitochondrian and the chemical state of some of its fixed reactants. The characteristic times observed for changes in morphology would appear to be considerably slower than the characteristic times of the chemical processes so that swelling and shrinking seem to depend on the steady-state concentration of one or more reactants. It is not yet necessary to suppose that a reversible cycle of morphological changes accompanies any single chemical reaction in phosphorylation.

A number of years ago Lucille Smith observed an interesting dependence of the light scattering of suspensions of Rhodospirillum rubrum fragments on light intensity and concomittant rate of reaction.[5] This result suggests that the granum may also manifest swelling and shrinking processes related to its chemical or photochemical processes. In the last two years Packer[6] has observed that the chloroplasts of higher plants manifest the same kind of light scattering behavior and he has shown that light scattering and photophosphorylation are closely related. More detailed studies of the morphological

process itself have been carried out by Itoh, Izawa and Shibata[7] using the electron microscope and the Coulter counter. They find that chloroplasts shrink in light with a large deformation of the chloroplast such that their axial ration increases from 1.7 to 2.6 on illumination. Again a close relationship between photophosphorylation and the new effect is shown. The evidence also suggests that chloroplast fragments and presumably grana are altered in shape in the process and may indeed be the primary level of the effect. The process of shrinking is rapid when it is produced by ATP, but much too slow to be related to individual photosynthetic reactions when shrinking is caused by illumination. The action spectrum is that of a chlorophyll with two major peaks, one at 435 mμ and the other at 680 mμ. There is, however, a pronounced and unexplained peak at 720-740 mμ. The effect is similar to that observed in mitochondria. The phenomenon attracts our attention to the possibility of a rather large structure-function relationship in these particles, but the results to date do little to convince us that photosynthesis or oxidative phosphorylation actually depend on these morphological changes. The latter may prove to be related to some other behavior of the organisms as for example the well-known ability of chloroplasts to adjust their positions within plant cells so as to improve their ability to collect light. We can do little to shed light on the fundamental nature of the morphological processes or to explain them at the present time. We can, however, review some of the present evidence for structure-function relationships in proteins and extrapolate from this to the possibilities for such relationships in the granum.

THE MECHANISM OF CERTAIN HEME-PROTEINS

At present there are three most promising explanations for the unusual adaptability, specificity and efficiency of the specific physiological reactions of proteins. It is probable that all three appear together or in pairwise combinations depending on the required protein function. The first of these is the passive organization of reacting molecules near ionizable groups of the protein to provide both specificity of binding and the needed reduction in activation free energy for enzymic processes. This is the oldest of the major proposals for enzymic mechanism but has been out of favor for some years. Recent experiments on new and more complex model systems, particularly by Bruice and his collaborators,[8] suggest that it may be more important than we have believed.

The second explanation is that the protein is so able to order dipolar and nonpolar groups of its own structure about bound reactants as to produce an electrostatic field highly favorable to an acceleration of the limiting step of an enzymic reaction chain. In a case simpler than an enzyme this effect can be illustrated by the oxidation-reduction potential of cytochrome c. In Fig. 1 is shown the reaction between the imidazole hemochrome and oxidized cytochrome c. The major effect of electron transfer in this reaction is to transport a positive charge from the octahedral hemichrome complex ion buried in the protein to the free hemichrome closely surrounded by water molecules. Experimentally the free energy change is large[9] and if one makes the reasonable guess that the dielectric constant within the protein is about 20, the entire value of the free energy change can be accounted for by the difference in dielectric constants at the positive charge. There is no doubt that the effect is important in biological reactions and it may be very important. However, Sullivan[10] has carried out a number of experiments which show that the effect is quantitatively less important than my simple calculation would indicate. The iron complex ion of cytochrome c has six ligand atoms. Four of these are porphyrin nitrogen atoms and the other two are thought to be

imidazole nitrogens. The latter are said to occupy the fifth and sixth ligand positions. Cytochrome c may be partially opened to replace the sixth-position ligand, presumably an imidazole group, by a cyanide ion. The charge situation is then reversed as compared with the intact protein since the resulting mixed iron complex of protein, porphyrin and cyanide ion has a negative charge in the reduced state and no net charge in the oxidized state. If the electrostatic effect is predominate, the potential should be shifted well over in favor of the oxidized state perhaps to -250 mv. yet it shifts only from +260 mv. to -62 mv. Hence the electrostatic effect cannot be the total explanation of the normal oxidation-reduction potential in this case and we must look to the fifth-position ligand or the porphyrin of the mixed complex for a more complete explanation. We are thus directed to the third possible explanation for the behavior of protein reactions.

The geometry of an isolated hemochrome or hemichrome complex is determined by the directed valences of the iron 3d orbitals. On incorporation of these complexes into the protein matrix other factors will influence the geometry of the complex. The local anisotropic electrostatic field can distort the ligands from the "zero-order" geometry of the isolated complex though it is hard to believe that the effect can be large. Of more importance, the thermodynamic factors of protein stability may be best satisfied, i.e., the lowest free energy of the total protein achieved, by distortion of the ligands from the zero-order geometry. The porphyrin may be bent or twisted in hydrophobic bonding and the fifth-position ligand and, in the case of cytochrome c, the sixth-position ligand, which is also coupled to the protein chain, can be reoriented as a consequence of local folding details to produce bending, twisting, compression or stretching of the nitrogen-iron bond.[11] It is beginning to look as though distortion of the porphyrin may not be too important in ground-state electronic processes of the heme proteins. In preliminary Faraday-effect experiments, Rosenberg[12] has found that there is no Faraday effect associated with the absorption bands of cytochrome c except with the very small band at 675 mμ and the delta band at about 315 mμ. According to present understanding of the Faraday effect these results mean that electronic states responsible for the prominent Soret and visible bands do not contain admixtures of 3d iron orbitals. There would thus appear to be no direct coupling between these π-system orbitals responsible for visible and Soret absorption and the iron 3d orbitals, though of course there is σ-bonding to the porphyrin nitrogen atoms. As Gouterman[13] has pointed out, the effect of metal ion on porphyrin spectra can be well explained by mixing of the iron p_π orbital with the π system. The chemical reactions, including the oxidation-reduction process, involve essentially pure 3d orbital. These observations suggest that the physiological reactions of iron are not strongly related to the porphyrin π system and are not much influenced by β-position substitution of the porphyrin or distortion of the porphyrin. It is not improbable that we shall come to understand the rather large changes in the position and intensity of the visible bands of porphyrin proteins during reaction to be the result in part of direct distortions of the π system of the porphyrin brought about by the forces due to the folding of the protein. Fleischer and colleagues[14] have recently demonstrated that the porphyrin plane can take on a number of conformations in different metal-porphyrin densities. These probably result from weak crystalline forces and lead us to believe that the porphyrin ring and thus the π system may be forced to take on many different conformations in different proteins. In general it can be anticipated that these distortions will have more influence on spectra than on the physiologically important electronic properties of the bound metal ion at least for heme proteins. However, the 3d orbitals of magnesium lie too high to be much involved in the reactions of

chlorophyll and a more direct connection between the particular conformation of the chlorophyll porphyrin π system and the electronic properties of the magnesium ion which depend on p orbitals may appear. It will be interesting to see what the Faraday effect is in the several bands of chlorophyll. Conceivably one of these bands contains an admixture of a charge transfer process in which an electron moves from the porphyrin π system to magnesium ion. According to the analysis of Witt, et al.,[15] the P700 pigment is photo-oxidized on illumination and magnesium could be the catalyst for this process.

This kind of consideration is still based on too few observations to allow us to eliminate porphyrin distortion as important in determining the electronic properties of heme iron. Nevertheless it does concentrate our attention on the fifth-position and sixth-position ligands of the iron complex as being primarily responsible for determining this electronic state. Thus aside from porphyrin effects the oxidation-reduction potential of cytochromes is not determined soley by the nature of the fifth- and sixth-position ligands, but also by the positioning of these ligands achieved in the tug of war between the directed valence of the 3d orbitals of iron and the tendency of the protein to fold to a state of lowest free energy. We have carried out several experiments to show that the oxidation-reduction potential and paramagnetic susceptibility of iron can be altered by alteration of the protein without change in nature of ligands,[10, 16, 17] and there is little reason to doubt that this is due to the distortion mechanism which thus plays an important role in establishing the necessary electronic properties for this particular physiological reaction. The mechanism also explains how the electronic properties of iron can be mutationally altered to become more suitable for evolving organism since changes of amino acid residues at key positions can alter the positions of the fifth and sixth ligands and thus alter the functional electronic properties of the iron atom.[11, 16] Electronic evolution is thus seen to be no more complicated than morphological evolution since it is based on the same mechanism. Experiments with chemical modification of single side-chains or pairs of side chains in chymotrypsin[18] convince us that the rate parameters of enzymes are evolved to their high levels of efficiency in the same way. Similarly the varying affinities of hemoglobins from different organisms for oxygen appear to be established in this way.[11]

We have been concerned with the possibility that dramatic changes in the folding of cytochrome c might occur on reduction of iron. Such changes might be expected either as a result of the elimination of the charge on heme iron or as a result of ligand reorientation since either factor could upset the order of conformational stability of the possible folded forms. We have mentioned that such refolding or shifts in folding might explain the shifts in the peaks of the visible spectrum. Reasonably satisfactory evidence, particularly from Okunuki's group,[19] shows that there is a difference in character of the protein in the sense that one form is much more resistant to proteolytic enzyme attack and to denaturation. However, we have been unable to provide definite evidence from viscosity experiments that any major change in size or shape occurs.[20] The viscosity experiments are, however, complicated and we have recently turned to dielectric-dispersion experiments. It has been possible to eliminate electroviscous effects which have made the method unreliable heretofore, and under such circumstances Yue has found that the oxidized and reduced forms of cytochrome c are very similar. The differences are small and about the magnitude expected from sedimentation experiments.[20] It is probable that there are differences in the proteins but there can be no large change in volume or shape. Somewhat similar conclusions can be reached with hemoglobin under physiological salt concentrations. A

number of years ago Takashima was able to show that large changes in dielectric relaxation time and dipole moment occurred on oxygenation of hemoglobin providing the experiments were carried out at very low salt concentrations.[21] At physiological salt concentrations no such effects could be observed in viscometry experiments.[22, 23] Muirhead and Perutz[24] have recently found on comparison of horse deoxyhemoglobin and human oxyhemoglobin crystals obtained from high-salt solutions that there is no detectable change in internal folding of the four quarters of hemoglobin, but that the β sub-proteins which form two of these quarters are positioned quite differently in the two proteins. If, as is thought, it is possible to consider this difference a true measure of the effect of oxygenation, we can conclude that the change in state of iron produced by oxygen binding results in significant alteration in quatenary structure. However, it is most important to note that this reorganization must also result from changes in internal folding since there is no other way to couple the state of the iron complex to the factors determining quatenary binding.[11] The resolution of the x-ray diffraction method for hemoglobin is only about 5 angstrom units so considerable variation in internal folding can occur without detection. There is little reason at present to believe that changes of much more than one or two angstrom units in some atom positions are required and the changes in geometry are detectable in the molecule as a whole only because the delicate balance of interactions among the quarters produces in the quatenary changes an amplification of the small internal changes. It is interesting to note that what may be all-important small shifts in the positions of many atoms during physiological reactions may not generally be detectable by x-ray methods even at highest resolution. It may be hoped that this is too pessimistic a view. The results of Takashima suggest that the largest changes in protein conformation occur at intermediate states of oxygenation rather than on formation of fully oxygenated hemoglobin. It may prove possible to crystallize these intermediates for x-ray studies.

The small shifts within the quarters of hemoglobin appear to be concentrated in the region of the fifth-position imidazole ligand behind the heme plane. Evidence largely from Antonini and Wyman and their co-workers[25] has drawn special attention to this part of the protein and, taken with the fifth-position imidazole linkage between iron and protein already discussed, presents reasonable qualitative explanations for most of the long-puzzling peculiarities of hemoglobin reactions. Only one of these need be discussed here. This is the so-called heme-heme interaction in which the replacement by oxygen of water at the sixth ligand position of the iron ion of one sub-protein increases the affinity for oxygen on the iron ion of a second sub-protein. At present this picture is best explained by the following steps: (1) oxygenation of one iron ion causes conformational readjustments in the region of its fifth-position ligand; (2) through the strong coupling between the sub-proteins these dislocations are spread to a second sub-protein to cause a readjustment of the fifth-ligand imidazole group of that sub-protein; (3) the change in the orientation of this ligand alters the electronic properties of the second iron ion to increase its affinity for oxygen. Thus the chemical reactions of two distinct sub-proteins are coupled by interactions through the conformations of the sub-proteins. The situation is actually more complicated than this since there are dependencies on the total structure of the protein. The total protein must have a very rigid conformation and we have begun to suspect that the high content of α-helix in these proteins is needed to establish rigidity. The α-helix is a very rigid structure[26] and undoubtedly stiffens the protein much as iron bars will stiffen a rubbery matrix. Even cytochrome c has a high helix content which we can currently estimate as forty per cent.

Rufus Lumry

THE STRUCTURE-FUNCTION RELATIONSHIP IN CHYMOTRYPSIN

Space prevents more than the briefest mention of studies from our laboratory[27] and that of Hess[28] at Cornell on the possibility of significant conformation changes in chymotrypsin during reaction. It is sufficient to say that although rather large changes in optical rotation and fluorescence yield, possibly indicating conformation change, can be detected on acyl enzyme formation or reaction of substrates under extreme conditions of pH, there is at present no evidence indicating any significant change in conformational parameters at the normal pH of activity which is 7.8. The data leads us to believe that there is a distinct conformation change away from the active conformation as pH is displaced away from 7.8 either toward basic or acidic values. Reaction with strong specific acylating agents or phosphorylating agents forces the protein back to its active conformation since it reacts only with the active form and stabilizes it. Although conformational parameters do shift at these pH values, the changes are not an essential aspect of the catalytic function itself. Further work may prove that small changes in conformation occur also at pH 7.8. We are in fact inclined to suspect the normal occurrence during catalysis of small rearrangements of conformation of the same magnitude as occur in hemoglobin and of equal importance. Unfortunately it would be very embarrassing to try to defend such a thesis on the basis of present facts.

Our experiments on hemoglobin and chymotrypsin and the x-ray studies of Muirhead and Perutz[24] are thus to some extent disappointing insofar as they do not provide positive evidence for a dynamic involvement of conformation in protein reactions.

POSSIBLE IMPLICATIONS OF EXISTING PROTEIN STRUCTURE-FUNCTION OBSERVATIONS FOR PHOTOSYNTHESIS

We have purposely selected certain of the results from studies of hemeproteins and chymotrypsin because of their possible bearing on the photosynthetic process. None of the work provides much confidence that major conformation changes or even significant chemical-mechanical interaction will be found essential in the photosynthetic process. On the other hand, the results do not exclude such processes and it would be unwise to ignore even the more extreme implications of such changes at present, particularly in view of the findings of Packer[6] and the Japanese group.[7] Let us therefore see what behavior might be predicted even though at this point in time several of our suggestions appear to be extreme.

There is first the possibility that the spectrum of pigment molecules and the electronic states of the functional atoms or bonds of these groups will be significantly determined by the forces of protein folding. The spectrum of the photon-collecting chlorophyll molecules of the bed is close to that of chlorophyll in solution and these molecules are probably little affected by their loose union with protein and lipoid. On the other hand the fluorescence quantum yield of these chlorophylls is at least tenfold lower than that of chlorophyll in homogeneous solution so that internal conversion does appear to be influenced by the bonding.[29] In green plants there are several different kinds of chlorophyll as indicated by spectral changes[30] and there is also the P700 pigment which may or may not be chlorophyll.[31] In view of what has been said above about heme spectra, it may be suggested that those forms which differ significantly from normal chlorophyll spectra attain their spectra as a result of distortions supplied by their respective proteins. We are particularly referring here to

the P700 pigment as a distorted form of chlorophyll. Some support for this idea comes from the studies of Izawa, Itho, Ogawa and Shibata[32] who found that treatment of isolated chromatophores of Rhodospirillum rubrum by the strong protein denaturing agent, dodecylbenzenesulfonate, shifted the 878 mμ peak of bacteriachlorophyll to 778 mμ. They attributed the effect either to the removal of a pre-existing interaction with proteins or with carotenoids, but it is probable that in either case the very large effect is dependent on protein denaturation. Frenkel has observed reversible shifts of the 878 mμ peak to shorter wavelengths in dilute detergent solutions.[33]

It has been shown that cytochrome c derives its iron electronic properties, notably the oxidation-reduction potential, in part from distortions of ligands enforced by the protein. Judging from the hemoglobin mechanism these properties may be further modified through strong interactions with neighboring proteins. This leads to the idea that the quantitative behavior of chloroplast enzymes as well as cytochromes and other pigmented proteins can be modified by structural factors to be different from behavior found in studies of the isolated proteins. There are, of course, possibilities for dielectric effects particularly a local reduction in effective dielectric constant as a result of high lipoid content though the ease of permeation of chloroplast fragments by charged molecules suggests that these effects are not very important. At present there seems to be no evidence that the c-type cytochromes of the photosynthetic system have unusual oxidation-reduction potentials. However, it is almost certain in view of the tightness of interaction among constituents that some fixed reactants derive the precise values of their electronic properties from distortion produced in forming the granum.

The most extreme suggestion provided by the work on heme-proteins is that of dynamic interaction of proteins in the granum or mitochondrian. As a result of strong aggregation conformational changes in one protein may be spread to nearby proteins to produce alterations in the chemical properties of these proteins. Changes in the chemical state of one protein could produce changes in its structure which are spread to nearby proteins. If the structure of the granum is sufficiently strong, whole chains of protein-supported single-step reactions could be coordinated in this way. Such a mechanism might, for example, coordinate the interaction between the two light acts to explain the puzzling observation that the quantum yield of photosynthesis is nearly independent of light color for wavelengths shorter than about 685 mμ.

An additional example in point is the mechanism of the collection of small packets of negative free energy to form a single packet of significant size to cause a needed chemical reaction. Muscle is a system designed to convert chemical energy into mechanical energy. Because of the principle of detailed balancing, at least in theory, it can be made to work backward to convert mechanical energy into chemical energy. It is believed that two one-electron processes are required in the mitochondrian to produce enough negative free energy for each molecule of ATP formed. One way to collect the required energy is to first convert the chemical energy of the two one-electron steps into mechanical energy of the phosphorylating enzyme. The latter then acts like muscle working backward to reconvert this energy in a single process into chemical energy of phosphorylation. This proposal is less unreasonable than it sounds since the free energies for phosphorylation are small and could easily be accommodated by minor distortion of proteins without any major refolding processes. On the other hand the single-packet energies involved in the production of oxygen in photosynthesis are relatively large and there must be considerable question as to the possibility that the large stock-piling of

small amounts of free-energy necessary for oxygen production can be brought about in this way.

In conclusion we may observe that the suggestions for mechanical mechanisms in photosynthesis are rather large departures from current approaches to mechanisms. There is in the literature abundant suggestions of conformational change during enzymic function (for pertinent examples see Ref. 34). These changes may prove to be real and large, but if our studies have any general significance, they will prove to be small on close investigation, though, of course, the small changes may be vitally important in the catalytic process. Enzyme chemistry is just beginning to move into the era of real mechanism study and it is probable that a good bit of work will have to be done on single enzymes before we have sufficient information to allow any profitable analysis of the detailed mechanism of photosynthesis. We do not yet know whether the integrity of the granum is essential for the process since the many failures to find reactions in detergent-treated preparations and in similar fractionation experiments are hardly proof of the need for integrity. The studies from the Enzyme Institute at Wisconsin suggest that only sub-particles of the mitochondrian need have structural integrity.[35] It is highly desirable that similar investigation of the granum be carried out.

We caution against the ready explanation of peculiar aspects of photosynthetic sub-processes as being "due to protein conformation change." On the other hand we are convinced that at the present time the best place to look for the unique basis of protein reactions is in conformational factors. We must continue to ask why Nature has made use of well-knit, highly organized particulate structures for both oxidative phosphorylation and photosynthesis. We must continue to inquire into the meaning of the experiments of Packer[6] and of Izawa, Itoh and Shibata.[7]

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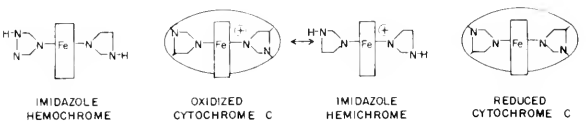
ELECTROSTATIC CONTRIBUTION TO THE FREE ENERGY OF THE OXIDATION-REDUCTION REACTION



$\Delta E_0 = 0.365$ V

$\Delta F^0 = -8.3$ kcal mole^{-1}

Figure 1.

IX. PATH OF CARBON AND ASSOCIATED METABOLISM

RECENT KINETIC STUDIES ON THE CARBON REDUCTION CYCLE

J.A. Bassham

The photosynthetic carbon reduction cycle (PSCR cycle) was proposed in essentially its present form about ten years ago [1], following some seven years of study by Calvin and coworkers, who used ^{14}C as a tracer to follow the path of carbon in photosynthesis. Since that time, it has been rather widely, though not universally, accepted as being the correct primary pathway of carbon dioxide reduction during photosynthesis. Studies from this laboratory [2] showed the PSCR cycle, or a cycle utilizing the same intermediate compounds, to be responsible for by far the greater part of the carbon dioxide reduction. Other later studies showed that carbohydrates are not unique as secondary products formed from the intermediates of the carbon cycle. For example, it was shown that the synthesis of carbon skeletons of amino acids could account for 30% or more of the rate of carbon uptake by the carbon reduction cycle during photosynthesis in Chlorella pyrenoidosa [3]. The pursuit of such quantitative studies led to the development of more sophisticated steady-state, steady tracer level techniques[2,3]. Development of these techniques in turn permitted us to restudy some kinetic properties of the PSCR cycle, and to investigate some puzzling facets of the ^{14}C labeling patterns which had been noted earlier in this and other laboratories.

Before presenting these questions and our efforts to find answers to them, I will discuss briefly the PSCR cycle, shown in Fig. 1. In this cycle, the first stable carboxylation product is 3-phosphoglyceric acid (PGA) [4]. All the remaining stable intermediates are sugar phosphates and diphosphates, with carbon skeletons from 3 to 7 carbon atoms in length.

There are four stages in the cycle: 1) Ribulose-1,5-diphosphate (RuDP) is carboxylated and gives two molecules of PGA; 2) PGA is reduced to triose phosphate; 3) A series of reactions convert five triose phosphate molecules to three ribulose monophosphate molecules; 4) The ribulose monophosphate molecules are then phosphorylated with ATP to give RuDP.

Carbon which enters this cycle as CO_2 is later "drained off" in the form of reduced carbon compounds such as PGA or sugar phosphates, by secondary photosynthetic pathways. Such pathways lead to the synthesis of fats, proteins, carbohydrates and other products. Part of these products are then used in the synthesis of new chloroplast structure while the remainder is "exported" to non-green parts of the plant cell, or to other parts of the plant in multicellular organisms.

The asterisks are intended to show the degree of labeling of various carbon atoms in the cycle following a short period (a few seconds) of photosynthesis with $^{14}CO_2$. Newly incorporated $^{14}CO_2$ becomes the carboxyl group of PGA and the unphosphorylated terminal carbon atom of phosphoglyceraldehyde and of phosphodihydroxyacetone. Condensation of these two triose phosphates with each other results in fructose-1,6-diphosphate labeled in carbon atoms 3 and 4. However,

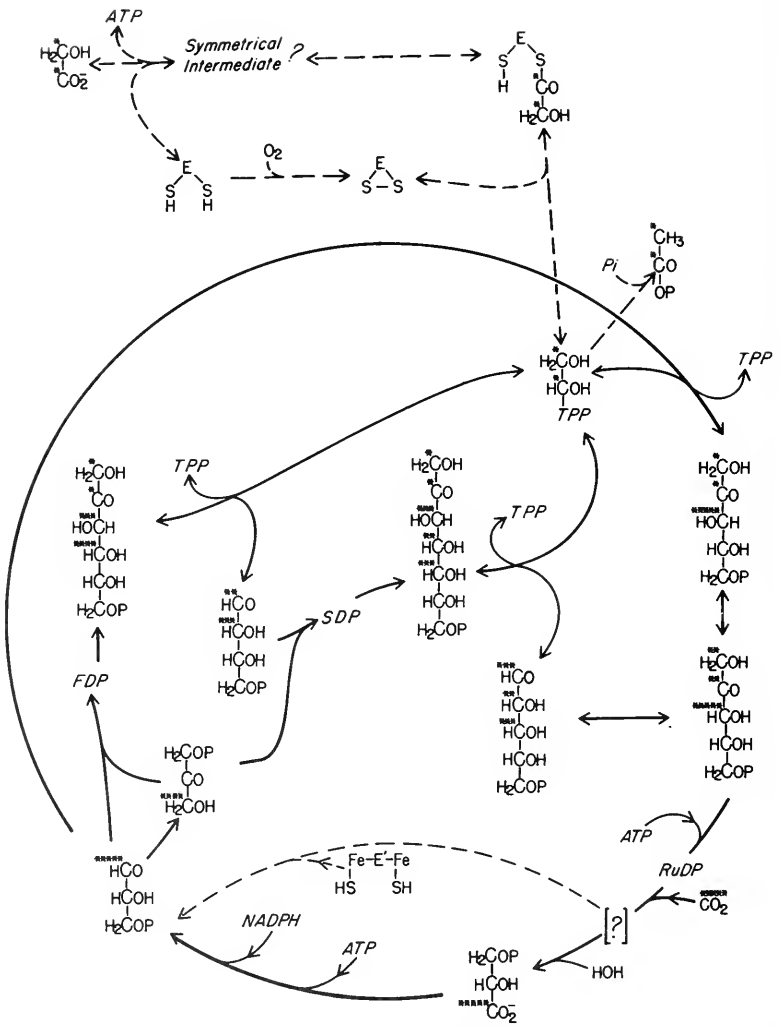


Fig. 1. The photosynthetic carbon reduction cycle (PSCR cycle). Abbreviations include: FDP, fructose-1,6-diphosphate; SDP, sedoheptulose-1,7-diphosphate; RuDP, ribulose-1,5-diphosphate; TPP, thiamine pyrophosphate; E, E', unspecified enzymes or proteins. The E'$(FeSH)_2$ symbolizes a reduced ferredoxin-type electron carrier with a potential of -0.4 v. The asterisks denote the order of labeling (not the accurate magnitude) of various carbon atoms of sugar phosphates, and other intermediates, following a short period (such as 10 sec) of photosynthesis with $^{14}CO_2$. Depending on pool sizes, particularly that of triose phosphates, asymmetry of labeling in hexose and heptose phosphates may be more or less than shown.

there is a higher label in carbon no. 4 than in carbon no. 3 if the equilibration of label between the two triose phosphates has been incomplete so that the specific activity of the glyceraldehyde phosphate is higher than that of phosphodihydroxyacetone.

A transketolase reaction on fructose-6-phosphate, and a subsequent condensation of the resulting tetrose phosphate with dihydroxyacetone phosphate leads to the formation of sedoheptulose diphosphate and of sedoheptulose monophosphate with the label predominantly in carbons 3,4 and 5 with more label in 3 and 5 than in 4. Such a labeling pattern was observed when leaves were exposed to $^{14}CO_2$ during photosynthesis for a second or less (1). This was evidence for the existence of differentially labeled triose phosphate pools and was so recognized at the time (1).

Another transketolase reaction on sedoheptulose-7-phosphate leads to the formation of a ribulose-5-phosphate labeled in carbon atoms 1,2 and 3. The carbon atoms 1 and 2 resulting from the transketolase reactions on fructose-6-phosphate and sedoheptulose-7-phosphate are considered to be in equilibrium with a pool of thiamine pyrophosphate-glycolaldehyde addition compound. Two-carbon moieties from this pool undergo a reversible reaction with glyceraldehyde phosphate to give molecules of xylulose-5-phosphate (labeled in carbon atom no. 3) which in turn is in equilibrium with a ribulose-5-phosphate pool and the ribose-5-phosphate pool mentioned above. Rapid reversible equilibration among these pentose phosphate pools and the thiamine pyrophosphate glycolaldehyde pool results in the feedback of labeling from carbon atoms 1 and 2 of ribose phosphate through ribulose phosphate, xylulose phosphate to glycolaldehyde thiamine pyrophosphate addition compound and thence to the number 1 and 2 carbon atoms of fructose-6-phosphate and sedoheptulose-7-phosphate. This is essentially the explanation which we have given previously (6) for the asymmetric labeling of hexose discovered by Kandler and Gibbs (7).

This rapid reversible equilibration is entirely to be expected, since the free energy changes associated with the transketolase and eperimisation reactions under steady state conditions probably are all in the range $F^S = +1.5$ to -1.5 Kcal. This close to equilibration, and with low activation energies for the reactions involved, the ratio between forward and back reactions is given by $F^S = -RT \ln f/b$ where f is the rate of the forward reaction and b is the rate of the back reaction. (5) At 25°C, $F^S = -1.37 \log f/b$, so that the back reaction is approximately 10% of the rate of the forward reaction.

Other reactions for the glycolaldehyde thiamine addition compound are its conversion by phosphoroclastic splitting to acetyl phosphate, and its oxidation to glycolic acid. Evidence for the acetyl phosphate formation by this path in photosynthesis is lacking. The stimulation of formation of labeled glycolic acid during photosynthesis with $^{14}CO_2$ at high O_2 levels has been demonstrated(8).

The Gibbs effect is often quoted as an argument against the correctness of the PSCR cycle (9). This effect, asymmetric labeling of hexose following short periods of photosynthesis with $^{14}CO_2$, consists of two parts: carbon atom no. 4 is more highly labeled than carbon atom no. 3, and carbon atoms 1 and 2 are more highly labeled than carbon atoms 5 and 6.

There is a difficulty in the explanation of the greater labeling of carbon atom 4 as compared with carbon atom 3. The pools of the two kinds of triose phosphate would have to be incompletely equilibrated with respect to carbon labeling. By the argument presented above, one would expect these two types of

triose phosphate to be in rather rapid equilibrium with each other. However, if the relation between phosphoglyceraldehyde and phosphodihydroxyacetone were different from that which exists in glycolysis, it is possible that the labeling of the dihydroxyacetone phosphate pool would not be reflected into the phosphoglyceraldehyde pool. Such a situation might exist if the phosphoglycolaldehyde moiety were bound to an enzyme and if its conversion to dihydroxyacetone phosphate resulted in a larger negative free energy change than is associated with the conversion of free phosphoglyceraldehyde to dihydroxyacetone phosphate.

The possibility that phosphoglyceraldehyde and perhaps erythrose-4-phosphate as well, may exist only in a form bound to the enzyme such as enzyme-S-CO-R is most attractive. It could explain why neither the triose phosphate nor tetrose phosphate are normally seen as labeled intermediates during studies of photosynthesis with $^{14}CO_2$. If the enzyme-bound phosphoglyceraldehyde were unable to react with inorganic phosphate to make phosphoryl-phosphoglycerate, as it does in glycolysis, the oxidation of triose phosphate to phosphoglyceric acid could be blocked. Such a block might be most advantageous to the photosynthetic mechanism, in that it would prevent reoxidation of newly formed sugar phosphates during short periods of darkness. I shall return to this point later.

It is also possible that the existence of such a bound form of phosphoglyceraldehyde, with a block towards its oxidation, could account for the reported lack of aldolase in some photosynthetic organisms (10,11,12). A reversal of the condensation reaction leading to fructose-1,6-diphosphate would give dihydroxyacetone phosphate and bound glyceraldehyde phosphate. Aldolase is known to bind dihydroxyacetone phosphate (13) as a Schiff base. Conceivably some organisms bind both triose phosphates so tightly that they can only be liberated by the condensation reaction leading to fructose-1,6-diphosphate. One might expect the pool of free dihydroxyacetone phosphate, commonly observed in studies of photosynthesis in leaves and in Chlorella with $^{14}CO_2$, to be missing from such organisms.

I would now like to focus attention on our more recent kinetic studies. In most of these experiments we establish a condition of steady state photosynthesis in which all reactions are proceeding at a constant rate and in which the intermediate pool sizes are maintained at constant levels (2,3). We then introduce $^{14}CO_2$ in a step function in such a way that the specific activity of the added tracer comes immediately to its final value and is maintained there during the course of the experiment. The addition of the tracer is accompanied by no other environmental change. Samples may be taken immediately following the addition of the $^{14}CO_2$ at frequent intervals and continuing through the time when intermediates of the carbon reduction cycle have become "saturated" with radiocarbon.

From the initial slopes of the labeling curves upon introduction of $^{14}CO_2$, we can calculate rates of flow of ^{14}C through specific intermediate pools. From the level of radioactivity in specific compounds when they are "saturated" we can determine the concentration of the actively turning over pool, by dividing the total radioactivity by the specific radioactivity of the $^{14}CO_2$ which is maintained constant and is measured. Following "saturation" of intermediates of the carbon cycle, we may vary some environmental factor, such as light or CO_2 pressure, and follow the changes in concentration in the actively turning over pools which are seen as changes in the total radioactivities of these pools (1,2,3,14,15).

J.A. Bassham

In the first of our more recent studies, we investigated the kinetics of the labeling of ribulose diphosphate and of PGA in vivo. We found that from the beginning of the period of steady state synthesis with $^{14}CO_2$ until saturation of the intermediates, the specific radioactivity of PGA was always considerably higher than that of RuDP (2). If the carboxylation of RuDP results in the formation of two molecules of PGA, then the carboxyl group of one of every two PGA molecules will contain the newly incorporated ^{14}C.

To test this model, we subtracted from the total PGA radiocarbon an amount of ^{14}C radioactivity which would correspond to the radioactivity expected in this carboxyl if the model were correct. Since this carboxyl group would rapidly saturate if the model were correct, this involved subtracting 1/2 x 1/3 = 1/6 of the saturation level of radiocarbon in PGA after about 30 seconds. The remaining ^{14}C which would have to be derived from the RuDP carbon atoms was compared with the ^{14}C in RuDP. The remaining, or residual, PGA carbon atoms were found to be labeled to a higher degree of saturation than the average of the five atoms of RuDP, suggesting that this model (2 molecules of PGA per carboxylation) was incorrect.

Using a different model in which only one PGA molecule was formed, and in which it was formed from the newly incorporated $^{14}CO_2$ and carbon atoms 1 and 2 of RuDP, a similar calculation showed that the residual carbon atoms of PGA were not labeled more rapidly than the average of the five RuDP carbon atoms until after 50 seconds. In this case there was no contradiction between model and data even after 50 seconds, since we know that carbon atoms 1 and 2 of RuDP are more quickly labeled than the carbon atoms 3, 4 and 5 (1).

From this data and reasoning, we concluded that the carboxylation of RuDP leads to only one molecule of PGA in equilibrium with the PGA pool. The other three carbon atoms from RuDP appear to have been converted either to a form of bound PGA not in equilibrium with the pool, or to some other molecule. We speculated that if the in vivo reaction were reductive, the other molecule might be triose phosphate.

It was noted earlier that the radioactivity in PGA does not always extrapolate to 100% at zero time (1). Sometimes such extrapolation gives 10-15% ^{14}C in sugar phosphates at zero time. This finding suggests that it is not only the three carbon moiety derived from carbons 3,4 and 5 of RuDP which may not be in equilibrium with the PGA pool. It appears that some of the PGA labeled with the newly incorporated ^{14}C in the carboxyl group is also bound, perhaps to an enzyme, and converted to sugar phosphates without freely equilibrating with the PGA pool.

The experimental evidence suggesting that the newly incorporated ^{14}C does not all pass through the free pool of PGA prompted us to perform further kinetic studies on the labeling of intermediates of the carbon cycle during photosynthesis. Light-dark transient studies were performed (16) under conditions of steady state photosynthesis. As in earlier light-dark transient studies (14,17), the level of PGA rose and the level of ribulose diphosphate fell when the light was turned off. However, under the more nearly steady state conditions used in the more recent study, the concentration of fructose diphosphate was higher than that of ribulose diphosphate and both diphosphates fell to zero in concentration in the dark with equal rapidity. Sedoheptulose diphosphate concentration also dropped, and the sum of the drops of these three diphosphates was not more than equal to the transient rise in PGA concentration. The levels of dihydroxyacetone phosphate and of fructose-6-phosphate

also declined, suggesting that some of the rise in PGA concentration might be due to a momentary reoxidation of sugar phosphates in addition to that resulting from continuing carboxylation of ribulose diphosphate coupled with a cessation of the reduction of PGA to sugar phosphate.

To investigate this point further, we undertook a kinetic study of the carbon labeling of cycle intermediates in preilluminated Chlorella. Following a period of steady state photosynthesis the light was turned off and $^{14}CO_2$ was immediately added without any alteration in the total level of CO_2 which was maintained at about 2%.

Some of the results of this experiment are shown in Fig. 2. There was a rapid

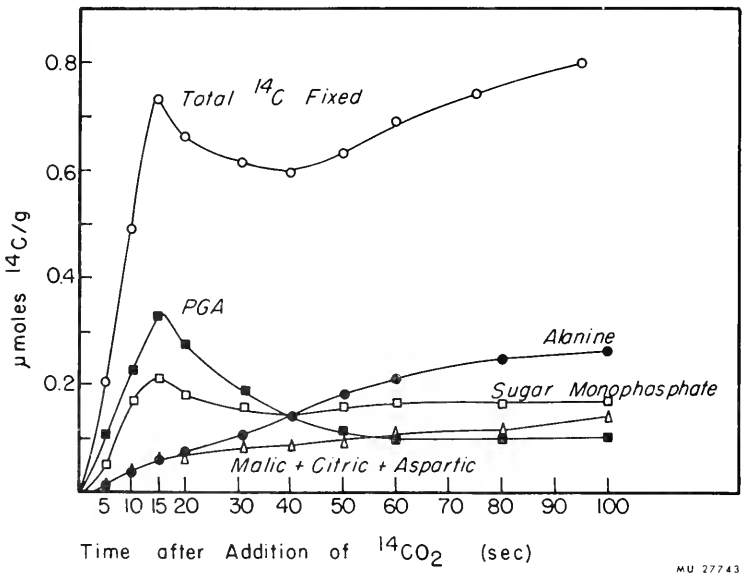


Fig. 2. Fixation of ^{14}C into compounds in Chlorella following preillumination. Sugar monophosphates include monophosphates of fructose, glucose, sedoheptulose, ribose and ribulose. "Total ^{14}C fixed" includes all radioactive compounds, including those on the origin, found on the chromatogram.

initial uptake of $^{14}CO_2$ during the first 15 seconds. This was followed by a temporary decrease in ^{14}C-labeling of the compounds. Presumably some of the newly incorporated ^{14}C was respired. After this drop, a slight loss of newly incorporated ^{14}C in such products as sugar monophosphate occurs during the period from 15-30 seconds. Thereafter, ^{14}C in these compounds remains essentially constant.

This ^{14}C labeling curve of sugar phosphates suggests that there is a mechanism which allows only a small amount of reoxidation of the newly photosynthesized sugar phosphates and then blocks further oxidation of these sugar phosphates by triose phosphate dehydrogenase type reactions. From the usual concept of the PSCR cycle in which PGA is reduced by triose phosphate dehydrogenase in the presence of ATP and NADPH, one might have predicted that once these cofactors were exhausted in the dark, the triose phosphate dehydrogenase reaction would be reversed and the newly formed sugar phosphates rapidly oxidized. Thus the result obtained here suggests once again the possibility that phosphoglyceraldehyde in the PSCR cycle exists only in an enzyme-bound form which cannot readily be oxidized. This hypothesis would require a corollary - the formation of the bound phosphoglyceraldehyde directly from an intermediate in the carboxylation reaction rather than from reduction of PGA.

Another interesting point seen in the preillumination study is the rapid rate of labeling of sugar monophosphates between 5 and 10 seconds. This rate is equal in magnitude to that of the labeling of PGA despite the fact that during this period the PGA carboxyl group in all probability can be no more than 20 - 30% saturated. (2) This rapid labeling of sugar monophosphates at a time when the PGA pool is only partly labeled appears to be rather direct evidence for the reduction of some bound form of newly incorporated $^{14}CO_2$ which is not in equilibrium with the PGA pool.

I have mentioned several bits of evidence which suggest the possibility that a carboxylation intermediate preceeding PGA might be converted directly to the sugar phosphates without equilibration of label between the intermediate compound and PGA. From the standpoint of chemical possibilities the most likely reaction for the accomplishment of this direct conversion would seem to be a reductive carboxylation reaction. However, the in vitro enzyme system for carboxylation of ribulose diphosphate accomplishes only the non-reductive dismutation to give two molecules of PGA. There is to date no enzymic evidence whatever for a reductive carboxylation. One must suppose that if such a reaction exists, it is mediated by some organized or multifunctional enzyme system which is most difficult to isolate intact from the living system.

In looking for reasons why such a system might be difficult to isolate, one could propose that the system is particulate or an enzyme of high molecular weight, and that the system is easily disrupted into soluble enzymes of smaller molecular weight which lack the necessary organization. A more plausible reason would be that in the in vivo system there is some direct link between the photochemical reactions which produce ATP and electrons, and the carbon reducing system. This link might then be lost when the system is isolated. We know that the green lamellar structures which carry out the photochemical reactions can be rather easily separated from the soluble carbon fixing enzymes (18).

When phosphopyridine nucleotide reductase (PPNR), discovered by San Pietro (19,20), was shown by Tagawa and Arnon (21) to be a non-heme iron protein of the ferredoxin type (22,23) it became a good candidate as the link between the photochemical apparatus and the carbon photosynthetic apparatus of photosynthesis. It has been generally supposed that chloroplast ferredoxin or PPNR mediates the transfer of electrons from the light reaction to NADPH, with the latter cofactor then being used to bring about the reductions of the carbon reduction cycle. However, ferredoxin is a stronger reducing agent than NADPH, being comparable to H_2. If it were used directly in the carbon reducing reactions, only one molecule of ATP per two electrons of reducing agent would be required rather than one and one half molecules of ATP per two electrons as would be the case with NADPH as sole reducing cofactor (5,24). I have proposed

that ferredoxin reduces an enzyme disulfide to disulfhydryl and that the enzyme disulfhydryl in turn functions directly in the reductive carboxylation reaction (24).

It is also possible that ferredoxin could function directly in the carbon reducing reaction. Valentine et al. (25) have shown that in the oxidation of pyruvate in Chlostridium acidi-urici, ferredoxin takes the place occupied by lipoic acid in other systems and mediates the electron transfer between pyruvate and NAD. Since ferredoxin contains free sulfide groups we may suppose that it is functioning here as a disulfide cofactor similar to lipoic acid, and that it is accepting an acetaldehyde moiety from the carboxylation of pyruvate. By analogy with the lipoic acid system, we may suppose that the intermediate is acetyl ferredoxin sulfhydryl which then reacts with inorganic phosphate to produce acetyl phosphate and ferredoxin disulfhydryl.

Possibly ferredoxin contains chains of FeS-FeS-FeS.. arranged so that two of the terminal sulfides have the same orientation as the disulfide of a lipoic acid molecule. At the other ends of these FeS chains, the Fe^{+3} atoms could accept electrons which would then be transported along the chain to the disulfide grouping which would then become disulfhydryl. In this way, the ferredoxin could function as a mediator between one electron and two electron oxidation reduction reactions.

Accepting electrons singly from the photochemical apparatus of the green cell, ferredoxin might transfer two electrons at the potential of hydrogen gas to the enzyme system responsible for the reductive carboxylation reaction. Without attempting to guess the detailed mechanism of this carboxylation reaction, we may nonetheless note that it bears a formal similarity to a reversal of the pyruvate oxidation discussed above.

With these thoughts in mind, we have attempted to investigate the kinetics of the carbon cycle of photosynthesis in the presence of added chemical agents which might interact with disulfide disulfhydryl systems. In one such study (26) we allowed Chlorella to photosynthesize in the presence of $^{14}CO_2$ for about 10 minutes under steady state conditions at pH 5.0. Without disrupting these conditions we introduced an amount of 8-methyl lipoic acid which gave an approximately 0.5 millimolar solution of this lipoic acid analog. Preliminary studies had shown that such an addition caused an immediate complete inhibition of oxygen evolution and CO_2 uptake.

The effect of the addition of this inhibitor upon the levels of various intermediates of the carbon reduction cycle and other photosynthetic products are shown in Figs. 3 and 4. The most dramatic effect is the immediate drop in the level of PGA which falls during the first 15 seconds after addition of the inhibitor to about 1/4 of its steady state value. At the same time, the levels of fructose diphosphate and of sedoheptulose diphosphate rise quite rapidly. Surprisingly, in view of the PGA effect, ribulose diphosphate undergoes only a small positive transient and then a slight decrease to a constant level. It was noted that lipoic acid itself also caused inhibition of photosynthesis, and in more recent but unpublished experiments we find that the effects of 8-methyl lipoic acid are reproduced by the same concentration of lipoic acid.

If the disulfide compound which we have added is accepting electrons from the light reaction and thereby keeping them from being used in carbon reduction, one might expect the resulting transient changes in the intermediates of the carbon cycle to resemble those seen upon turning off the light. It is clear

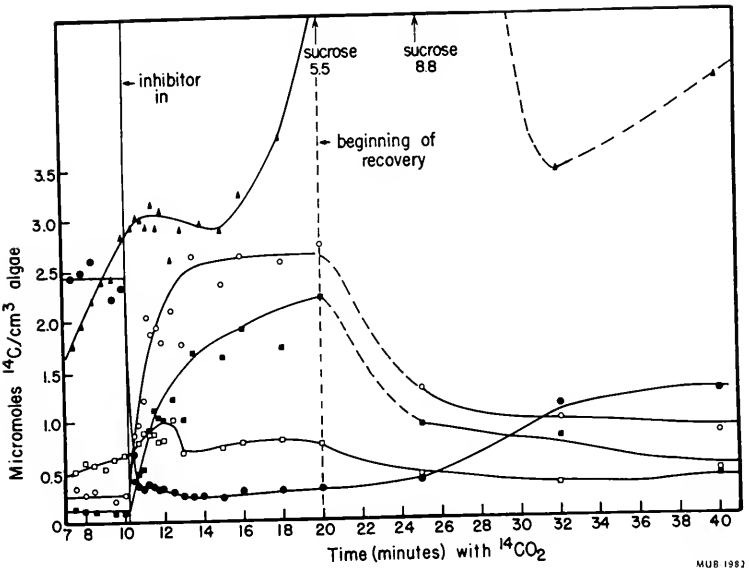


Fig. 3. Effects of 8-methyl lipoic acid on labeling of compounds with ^{14}C during photosynthesis with $^{14}CO_2$. ▲ Sucrose, O fructose-1,6-diphosphate, ■ sedoheptulose-1,7-diphosphate, □ glucose "diphosphate", ● PGA.

that this is by no means the case, since PGA drops instead of increasing and since ribulose diphosphate does not drop immediately to zero concentration. The extremely rapid drop in PGA concentration requires either that the carboxylation reaction leading to the formation of PGA be inhibited or that the reduction of PGA be greatly accelerated. The latter possibility is not borne out by the transient behavior of other carbon fixation products. Although there is a rise in the fructose and sedoheptulose diphosphates, there is a corresponding drop in their monophosphates, which would not be expected from an acceleration of PGA reduction.

Thus we are left with a dramatic inhibition of reactions leading to PGA, but without a concomittant increase in the level of ribulose diphosphate which we would expect if the carboxylation of ribulose diphosphate to give PGA were stopped. Perhaps in addition to the inhibition of the carboxylation of ribulose diphosphate to give PGA, there is a subsequent inhibition of the formation of ATP. This would prevent the level of ribulose diphosphate from rising.

Another possibility is that in the in vivo system phosphate groups from the no. 1 carbon atoms of fructose and sedoheptulose diphosphates are transferred to ribulose-5-phosphate to make ribulose-1,5-diphosphate. This would account for the apparent inhibition of the conversion of the hexose and heptose diphosphates to their monophosphates. Whatever the precise explanation of the various effects of the disulfide compounds may prove to be, it is clear that these substances interfere suddenly and dramatically with the carbon reduction cycle.

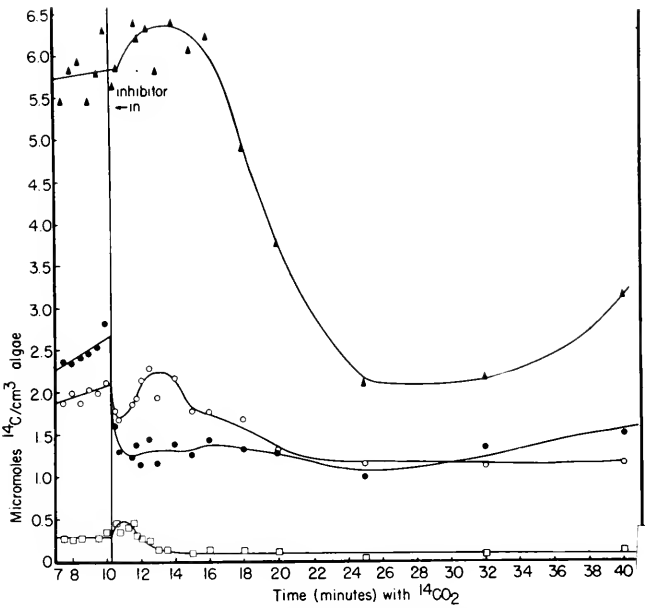


Fig. 4. Effects of 8-methyl lipoic acid on labeling of compounds with ^{14}C during photosynthesis with $^{14}CO_2$. ▲ Glucose-6-phosphate, ● sedoheptulose-7-phosphate, ○ fructose-6-phosphate, □ ribulose-1,5-diphosphate.

The immediacy of their effects makes it seem plausible to suppose that they are interferring in some way with electron transport by compounds with disulfide groups.

We have recently carried out experiments under our steady state conditions of photosynthesis with $^{14}CO_2$ in which CO_2 in air is suddenly replaced by nitrogen. After about 10 minutes the $^{14}CO_2$ in air is again added to the photosynthesizing algae. The results of these experiments are shown in Figs. 5 and 6. The drop in the level of PGA and the rise and fall in the level of ribulose diphosphate are similar to those noted by Wilson and Calvin (15). The behavior of the intermediates on addition of $^{14}CO_2$ again is quite interesting. As would be expected, the level of ribulose diphosphate falls while that of PGA rises as the carboxylation reaction resumes. What is intriguing is the fact that the level of fructose diphosphate rises as rapidly as that of PGA during the first few seconds. This is an additional bit of evidence for the direct conversion of the carboxylation intermediates to sugar phosphate without equilibration through the PGA pool.

Another interesting point is the fact that fructose diphosphate and sedoheptulose phosphate rise and then fall after the readdition of the $^{14}CO_2$. Why do they fall? One could argue that the initial carboxylation reaction is faster than usual because of the high level of ribulose diphosphate, and that once the ribulose diphosphate has been depleted, the carboxylation reaction becomes slower. However, the level of PGA which continues to rise for many minutes belies this argument. Perhaps the explanation is to be found in the previously

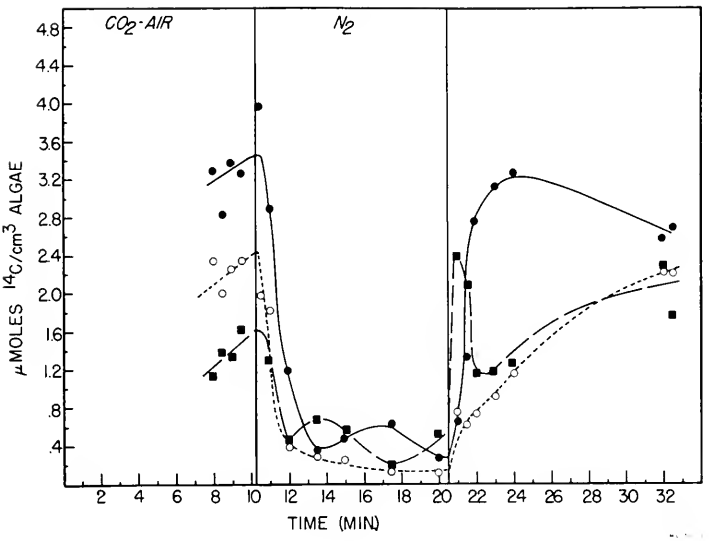


Fig. 5. Effects of sudden removal and readdition of CO_2 on levels of PSCR cycle. Sugar monophosphates. ● Glucose monophosphate, ○ sedoheptulose monophosphate, ■ fructose monophosphate.

suggested transfer of C_1 phosphate groups from fructose diphosphate and sedoheptulose diphosphate to ribulose monophosphate. This transfer which might initially be inhibited by the high level of ribulose diphosphate would accelerate as the level of ribulose diphosphate decreases.

In summary, while recent kinetic studies of the carbon reduction cycle of photosynthesis confirm the cycle in essentially the form in which it was proposed ten years ago, there is a scattering of evidence which suggests that the in vivo cycle might vary somewhat in its mechanistic details from the cycle which was proposed at that time. First there is a variety of kinetic evidence which suggests the direct conversion of the carboxylation intermediate compound to the level of sugar phosphate without complete equilibration with the free pool of PGA. Secondly, there is some indication that when the light is turned off there is only a partial brief reoxidation of sugar phosphate via triose phosphate dehydrogenase and that this period is followed by a block on this reoxidation. Third, some experiments suggest the possibility of direct transfer of phosphate groups from the no. 1 carbon atoms of hexose and heptose diphosphates to ribulose monophosphate. Fourth, the dramatic effects of disulfide compounds on the carbon reduction patterns, and other biochemical considerations argue for a role of disulfide compounds, perhaps including ferredoxin, in mediating a reductive carboxylation of ribulose diphosphate to give sugar phosphates directly. A separate but related possibility is that some of the newly incorporated carboxyl carbon is also reduced to the level of sugar phosphate without an opportunity for equilibration with the free PGA pool. Such a direct

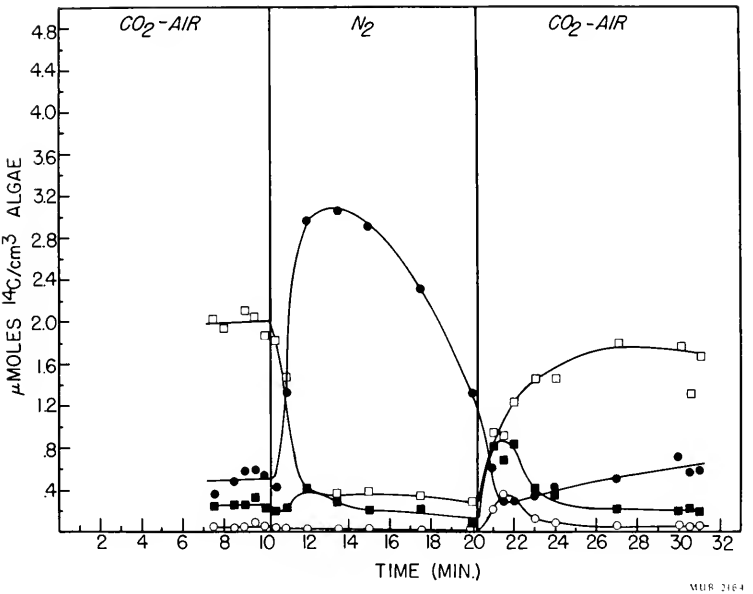


Fig. 6. Effects of sudden removal and readdition of CO_2 on levels of PSCR cycle. Sugar diphosphates and PGA. ● Ribulose-1,5-diphosphate, □ 3-phosphoglyceric acid, ■ fructose-1,6-diphosphate, ○ sedoheptulose-1,7-diphosphate.

reduction of newly incorporated carbon to the level of bound triose phosphate would be required if we are to invoke the bound glyceraldehyde phosphate moiety as an explanation for the asymmetry of the hexose phosphate labeling and the slow labeling of carbon atom 4 of sedoheptulose phosphate.

The mediation of the reactions of the cycle may be accomplished by a multifunctional, organized enzyme system. This system should be closely linked to the photochemical apparatus, perhaps by such a compound as PPNR (chloroplast ferredoxin). The system should include enzyme "handles" for holding some of the intermediate compounds in a bound form. Bound forms might include glycolaldehyde, bound to thiazolium groups in transketolase reactions, and aldehyde moieties bound to disulfide/disulfhydryl groups in carboxylation, condensation and epimerisation reactions.Attempts to isolate active enzymes from the cell may result in the loss of the organization and primary enzymic activities. Residual enzymic activities found in the soluble isolated protein could mimic the reactions of the intact system. Such activities might well be variable and in many cases inadequate to accomplish the reactions of the PSCR cycle at anything like the in vivo rate.

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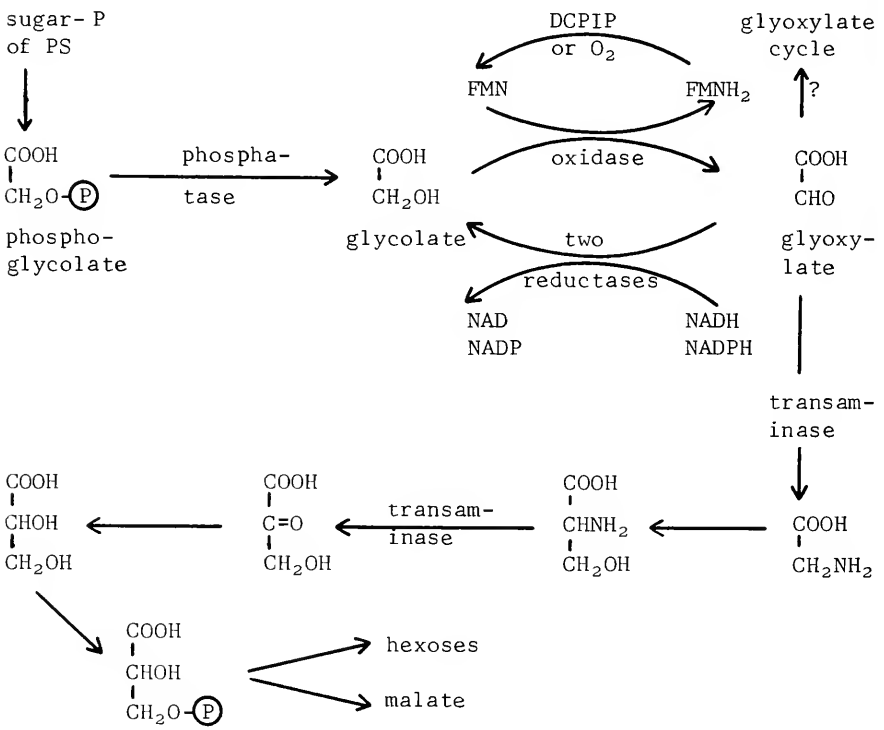
GLYCOLATE PATHWAY

N. E. Tolbert

During $C^{14}O_2$ photosynthesis, glycolate and its derivatives become labeled. The sequence of reactions producing this pattern of labeling is referred to here as the glycolate pathway (1). These reactions are thought to be a metabolic process which occurs after fixation of the CO_2 by the photosynthetic carbon cycle. The following formulation of this pathway and its relation to other metabolic processes is based on information in numerous publications since 1950.



N. E. Tolbert

Magnitude of the Glycolate Pathway

To obtain a picture of the quantitative significance of the pathway, amounts of C^{14} in the reservoirs of phosphoglycolate, glycolate, glycine and serine should be considered together. The glycolate pathway is blocked by α-hydroxysulfonates which inhibit glycolate oxidase (2) and by isonicotinyl hydrazide, an inhibitor of transaminase (3). When either inhibitor is used with in vivo photosynthesis a remarkable accumulation of C^{14} in glycolate products results, for over 50% of the fixed $C^{14}O_2$ in 10 minutes may be present in glycolate and glycine. According to Zelitch and Whittingham this accumulation occurs without a decrease in the rate of CO_2 fixation. However, Asada and Kasai (4) observed over 70% inhibition of photosynthesis by 0.01 M α-hydroxysulfonates. Such results suggest that much of the C^{14} newly fixed during photosynthesis is metabolized by the glycolate pathway.

Even without inhibitors a very large proportion of the fixed CO_2 is found in glycolate. Warburg and Krippahl (5) reported that Chorella during a 10 min. period converted 92% of the fixed CO_2 into glycolate. We have not achieved such yields with Chlorella, but have approached such values with Chlamydomonas. Also, we can treat chloroplasts so that 75% of the fixed $C^{14}O_2$ will appear in glycolate. Experiments were reproducible from day to day with Chlamydomonas, but over periods of months values were obtained which ranged from 25 to 75% of the total C^{14} which accumulated in glycolate products. Many plant materials produce much less glycolate products, but they all produce significant amounts in brief periods of photosynthesis. It should be emphasized that glycolate accumulation occurs in particularly large amounts with young tissue or with algae in the log phase of growth. Algae of mixed ages can give misleading results. Older tissue or tissues partially inhibited by water deficits and mistreatment will incorporate much of their C^{14} into sucrose. With care and with young tissue 50% of the C^{14} fixed during a 10-minute photosynthetic period in air will be found in glycolate products when one uses wheat, barley, sugarbeets, Ankistrodesmus, Chlamydomonas and, sometimes, Chlorella.

Distribution of C^{14} in Components

Calvin's group showed that glycolate produced during photosynthesis had the same specific activity in both carbon atoms (6). Thus all subsequent products of the glycolate pathway should be uniformly labeled. We found that serine after 4 seconds of photosynthesis was uniformly labeled (1) even though the phosphoglycerate from the same experiments had 20 times more C^{14} in the carboxyl carbon than in the α and β carbons. Therefore the serine

could not have been formed directly from phosphoglycerate, but could have been formed from glycolate. The glycolate pathway results in the production of uniformly labeled glycerate (1), which in turn, produces uniformly labeled hexoses, instead of 3,4-labeled hexoses as expected by the photosynthetic carbon cycle. The rapid incorporation of specific C^{14}-labeled glycolate, glycine and serine into glycerate and sugars and the corresponding labeling patterns in these products have been sufficiently documented to establish this part of the sequence (1, 7, 8, 9). Sucrose synthesis by this pathway occurs in the light; in the dark glycolate is metabolized to malate. However several anomalies still exist. Free glycerate is produced by the glycolate pathway. Plant enzymes are known for this synthesis, since a glyoxylate-serine transaminase produces hydroxypyruvate (E. R. Waygood, personal communication) which in turn is reduced by D-glycerate dehydrogenase (10). Nevertheless, the bulk of the free glycerate in all plants examined has been carboxyl labeled during photosynthesis (1, 11), even though the large serine reservoir is uniformly labeled. Thus the free glycerate pool in some plants is derived mostly from phosphoglycerate, although uniformly labeled glycerate is produced by the glycolate pathway. Wang and Burris (8) have also observed considerable phosphoserine whose function is unknown except that it could lead to uniformly labeled phosphoglycerate.

An exception to the above C^{14} distribution patterns was the label found in serine produced by isolated chloroplasts. Only a small amount of serine was formed during CO_2 fixation by whole chloroplasts, but it was carboxyl labeled and probably came directly from phosphoglycerate (W. Chang and N. E. Tolbert, unpublished). The same isolated chloroplasts produced more glycine than serine, and the glycine was uniformly labeled as if it came from glycolate. Glycine conversion to serine did not occur readily in chloroplast preparations. Thus, there seems to be two serine pools, one in the chloroplast which is formed from phosphoglycerate and one elsewhere which is formed by the glycolate pathway.

Origin of Glycolate

Phosphoglycolate: The amount of phosphoglycolate is difficult to determine for it nearly cochromatographs with phosphoglycerate (12). Another reason for inaccurate estimation of phosphoglycolate is the presence of an active phosphatase which hydrolyzes this ester (13). This phosphatase is stable in dilute methanol (14), acetone and ethanol, and often it may not be destroyed during killing procedures. When isolated chloroplasts were treated to produce glycolate products, a large amount of C^{14} was found in phosphoglycolate (15, 16) in part because the chloroplasts had lost much of the phosphatase. Because phosphoglycolate is a $C^{14}O_2$

fixation product and a specific phosphatase for its hydrolysis exists, it seems probable that phosphoglycolate is the immediate precursor of glycolate. Past theories for glycolate biosynthesis should be reconsidered to include the phosphate ester.

C_1 plus C_1 condensation: Synthesis directly from CO_2 would produce uniformly labeled glycolate as is actually observed. Proposals for such a pathway have not yet been supported by convincing data (17, 18).

Carbon cycle: It has long been postulated by many of us that glycolate arose from a sugar phosphate of the photosynthetic carbon cycle, but space limitation does not permit an extensive review of this literature. Bassham, Benson, Calvin, et al. (19) demonstrated that carbons 1 and 2 of RuDP, hexose phosphate and sedoheptulose phosphate were uniformly labeled and could thus give rise to a uniformly labeled C_2 moiety. Ribose-1-C^{14} added in vivo and ribose-5-phosphate-1-C^{14} added in vitro gave rise to glycine-2-C^{14} (20, 21, 22). Such results are consistent with cleavage between C_2 and C_3 of a keto sugar phosphate so that carbon 2 of the sugar became the carboxyl of glycolate. These reactions can be modeled after TPP linked transketolase, except that a free C_2 piece would be liberated rather than transferred. Several points about this theory await clarification. The yields of glycine from ribose-5-phosphate have been small, and no one has detected the cleavage enzyme. Small yields with crude enzymes are suspect, particularly since RuDP nonenzymatically decomposes into a multitude of products including C_2 pieces. In Bradbeer and Racker's abstract (23) on the production of free glycolate from fructose-6-phosphate in the light by chloroplasts, the yields were also very small. Since phosphoglycolate appears to be the precursor of glycolate, the sugar should be a diphosphate, which is not known to be cleaved by transketolase. Thus phosphoglycolate formation might arise from a cleavage of xylulose, fructose or sedoheptulose diphosphates. RuDP should not be a precursor, because it does not have the necessary transconfiguration of the hydroxyl groups between carbons 3 and 4 as is generally required by aldolases, transaldolases and transketolases. Phosphoglycolaldehyde might be a precursor for phosphoglycolate. Although aldolase has been shown to catalyze the formation of xylulose diphosphate from glycolaldehyde phosphate plus dihydroxyacetone phosphate (24), the reverse of this reaction would produce the C_2-phosphate from carbons 4 and 5 of the pentose. During photosynthesis these two carbons are also equally labeled with C^{14}.

Recently we have examined two facets of this problem. A

glycolaldehyde phosphate dehydrogenase system producing NADPH, ATP and phosphoglycolate was reported in abstract (25). However, the results have not been consistently reproducible. No data exist which implicates xylulose diphosphate in photosynthesis. When the ribulose diphosphate area from paper chromatograms of $C^{14}O_2$ photosynthesis experiments was treated with acid phosphatases free of 3-epimerase, 12 to 25% of the C^{14} followed xylulose on ion exchange borate columns (G. Orth and N. E. Tolbert, unpublished). The formation of xylulose diphosphate during photosynthesis needs to be confirmed by a method which will prevent epimerization of RuDP during isolation and identification. The problems of phosphoglycolate synthesis, insufficiency of aldolase in chloroplasts and even whether ribulose diphosphate is the only precursor for CO_2 fixation, all seem to be interrelated and certainly require further investigation.

Glycolate Excretion

By algae: When photosynthesizing Chlorella or Chlamydomonas utilize $C^{14}O_2$, nearly all of the glycolate-C^{14} produced is found in the supernatant fluid as the main C^{14} product therein and not in the cells (5, 26, 27, 28, 29). Whittingham's group has reported excretion rates for Chlorella of 0.35 μg glycolate per μl cells per hour, and Warburg has reported 6.8 μg per μl cells per hour. Originally, we had found a maximum glycolate accumulation of 4 mg per liter for actively growing cultures. Old cultures contain little glycolate in the medium. Fogg (30) has extended the concept that glycolate excretion by phytoplankton may represent a reservoir of chemical energy for the plankton which is built up during active photosynthesis for use at other times.

In this work it is necessary to separate the algae from the supernatant fluid by centrifugation or by filtration on a 1 cm thick celite pad. The latter process requires about 15 to 30 seconds. The glycolate-C^{14} pool from photosynthesis was metabolically active as judged by the fact that it had been rapidly formed and was partially converted to glycine and serine. When the algae were placed in the dark after $C^{14}O_2$ fixation, the glycolate-C^{14} was located inside the cells for it was not removed from the algae by filtration. The cells will rapidly absorb free glycolic acid at pH 3, but glycolate excretion occurs at neutral pH values. Moreover, if one were to add glycolate-C^{14} externally to such algae cultures in the dark or light, the rate of entry is much slower than the excretion and in fact at pH 6 several hours of light are required for glycolate-C^{14} uptake (26, 28). Thus glycolate-C^{14} produced by photosynthesis and glycolate-C^{14} added externally are not metabolically equivalent, even though glycolate-C^{14} produced by photosynthesis could be captured outside the

cells by filtration. This anomaly almost forces us to speculate that part of the glycolate produced by photosynthesis is not freely excreted, but that it is in or on the Chlorella in a position where it can be removed during filtration. In Mn deficient cells glycolate excretion does not occur (28), presumably because glycolate is all converted to glycine and serine.

By chloroplasts: When chloroplasts are killed rapidly about 5 to 10% of the total C^{14} fixed in 10 or 30 minutes is present as products of the glycolate pathway and the remainder are sugar phosphates. However, when the chloroplasts are filtered and washed with saline solution on the 1 cm celite pad for 30 to 90 seconds in order to separate them from the supernatant medium, 50 to 70% of the total fixed C^{14} was found in the supernatant medium as phosphoglycolate and glycolate, and the sugar phosphates had nearly disappeared (15, 16). The sugar phosphate esters were not themselves lost from the chloroplasts, but during filtration they were converted to phosphoglycolate and excreted as such. For some reason the filtration seems to have upset an equilibrium to create this change. During $C^{14}O_2$ by chloroplasts addition of α-hydroxysulfonates also increases the amount of glycolate-C^{14}, as if glycolate metabolism normally occurs in the chloroplasts even though it does not accumulate (22). Again when glycolate-C^{14} was added to chloroplasts it was not rapidly taken up nor metabolized.

These chloroplast experiments prove that the system for phosphoglycolate synthesis resides in the chloroplasts. In addition they indicate that a route for glycolate synthesis is from the sugar phosphates of the carbon cycle and not by a C_1 plus C_1 condensation. The production of phosphoglycolate indicates that it is the precursor of glycolate.

Some free glycerate is excreted by spinach chloroplast but it generally accounts for only a small part of the C^{14} (15). The conditions responsible for glycerate loss by the chloroplasts are not as reproducible as those for glycolate excretion. It must be emphasized, though, that free glycerate is excreted from the chloroplasts, while phosphoglycerate is the major product inside the chloroplasts. Phosphoglycolate phosphatase will not hydrolyze phosphoglycerate, but perhaps there is some other phosphatase within the chloroplasts which is responsible for phosphoglycerate hydrolysis and excretion.

Enzymes of the Glycolate Pathway

Phosphoglycolate phosphatase is apparently specific for this phosphate ester (13). It is ubiquitous in the green parts of plants, for it is not in roots or etiolated leaves (Y. Yu, N. E.

Tolbert and G. Orth, unpublished). The activity of this phosphatase is equal to or exceeds that of other phosphatases in the leaf. For example phosphoglycolate is hydrolyzed 5 times faster by wheat sap at pH 6.3 than most other phosphate esters (e.g. 3-phosphoglycerate). About 9% of the phosphoglycolate phosphatase activity from spinach, tobacco or swiss chard leaves remains with unwashed chloroplasts. Once washed chloroplasts have 2% the total enzyme, and if washed chloroplasts are then put in water, the rest of this phosphatase goes into solution, while the phosphatase responsible for hydrolysis of fructose-1,6-diphosphate remains with the particles. Preparation of chloroplasts in glycerol yields similar results. The phosphatase is certainly not tightly bound in the chloroplasts.

Phosphoglycolate phosphatase is stable at pH 4, and it has been purified 200-fold by acetone and ethanol fractionation and DEAE-cellulose. The stability of the enzyme from wheat is dependent upon the natural association with it of cis-aconitate. If the factor were removed by Sephadex, the enzyme is stable at 0° for only a short period of time. Stability at 40° is restored only by cis-aconitate, citrate or isocitrate. Our most purified preparation of the enzyme had both phosphatase and ATPase activity. The ATPase was less active than the phosphatase. The enzyme also forms methyl or ethyl phosphates when flooded with the alcohol, but this is typical of phosphatases. In addition the protein rapidly incorporated P^{32}-orthophosphate in an ATP dependent reaction. This latter reaction was heat stable, and the protein was not precipitated by heat. These properties are in part characteristic of certain phosphatases or ATPase which may be involved in transport phenomena. Our published procedure for isolating this enzyme from tobacco leaves involved $(NH_4)_2SO_4$ treatment which destroyed the ATPase and changed the pH optimum of the remaining phosphoglycolate phosphatase from 6.3 to 5 or less (13). The enzyme from most other leaves is inactivated by $(NH_4)_2SO_4$.

Like the phosphatase, active forms of glycolate oxidase (31) and a specific glyoxylate-serine transaminase (E. R. Waygood, personal communication) are nearly absent in etiolated leaves and are formed during greening. This characteristic is presumptive evidence that they are associated with photosynthesis. Glycolate oxidase also may be loosely associated with the chloroplast (22, 32, 33). There is also a NAD linked glyoxylate reductase which is probably cytoplasmic (34) and another glyoxylate reductase which is NADP linked and located in the chloroplast (35). Further studies concerning the formation or activation of these enzymes during greening of the plant should be pertinent to an understanding of chloroplast structure and its association with the rest of the cell.

N. E. Tolbert

Photosynthetic Requirements For Glycolate Synthesis

Light and effect of CMU and Cl-CCP: Very high light intensities are required for maximum glycolate synthesis and excretion (5, 26, 29). When Chlorella were grown continuously in fluorescent red light they incorporated little C^{14} into glycolate during 10 min. exposure to $C^{14}O_2$ in either red or white light, but they still labeled glycine and serine (Table 1). They acted like Mn deficient algae. When Chlorella were grown in blue light, much of the C^{14} was fixed into glycolate and it was excreted. Note also that algae grown in red light labeled aspartate but not when grown in blue light. Cayle and Emerson (36) have also reported greater C^{14} specific activity in glycine and serine during photosynthesis in blue light.

Table 1. Influence of red and blue light on $C^{14}O_2$ fixation.

Light during growth	PS	Total C^{14} c/s	% Distribution of C^{14} Sucrose	Glycolate	Glycine + serine	Aspartate
red	red	1570	1.8	2.0	27.0	8.9
red	blue	1570	2.6	3.7	28.2	8.4
blue	blue	1980	1.1	21.7	22.6	1.1
blue	red	1520	1.1	15.5	30.1	1.1

Table 2. CMU and Cl-CCP inhibition of $C^{14}O_2$ fixation in 10 min. by Ankisterodesmus.*

Inhibitor	Total c/s fixed x 10^6	% Distribution of C^{14} Glycolate products	P-esters	Sucrose	Citric** acid cycle
water	1.21	11.2	26.2	11.2	44.0
5 x 10^{-6}M CMU	0.29	5.9	14.1	6.2	63.3
5 x 10^{-5}M CMU	0.05	tr	tr	0	95.7
3.3 x 10^{-6}M Cl-CCP	0.39	14.7	36.7	15.8	25.0
1 x 10^{-5}M Cl-CCP	0.09	13.1	27.5	28.7	22.6

*Nearly similar results were obtained with Chlorella.
**Almost entirely aspartic, glutamic and malic.

CMU inhibits O_2 production during photosynthesis. As indicated in Table 2, the synthesis of glycolate products, phosphate esters of the carbon cycle and sucrose were reduced proportionally by CMU. However, with CMU a much greater percent of the fixed C^{14} appeared in glutamic, aspartate and malate. In comparison Cl-CCP, which inhibits photophosphorylation, preferentially reduced C^{14} labeling of aspartate, glutamate and malate. CMU and Cl-CCP did not inhibit glycolate phosphatase in vitro.

CO_2 concentration: Optimum glycolate accumulation and excretion during photosynthesis occurs at low CO_2 partial pressures of around 0.1% (5, 26, 28, 29). Below 0.1% CO_2 the photosynthetic rate is limited by CO_2 availability. Thus the total amount of glycolate synthesis is reduced, but the % of the fixed C^{14} in glycolate products is not reduced (Table 3) (W. Snyder and N. E. Tolbert, unpublished). It seems significant that glycolate secretion in large amounts must occur during photosynthesis in nature where the CO_2 partial pressure is so limiting. Above about 0.4% CO_2 the amount of glycolate which accumulates is reduced to much lower values (Table 3) (28, 29). At higher CO_2

Table 3. Effect of CO_2 concentration on glycolate products in beet leaves.

CO_2 in air %	Total C^{14} fixed c/s x 10^{-3}	Glycine plus serine * % C^{14}	Glycine plus serine * c/s x 10^{-3}
0.005	32	25	8
0.03	103	25	26
0.3	126	5	6
1.0	63	3	2

* Significant amounts of glycolate did not accumulate.

concentrations much more sucrose is made, but some newly fixed C^{14} still appears in glycine and serine. Such experiments suggest that the glycolate pathway was inhibited by higher CO_2 concentration. Since glycolate-C^{14} is rapidly incorporated into sucrose (1, 7), it is possible that the reservoirs of the glycolate products may simply be turning over more rapidly at higher CO_2 concentrations thereby decreasing rather than increasing in size.

A theory for the increased percentage of C^{14} in glycolate during photosynthesis at low CO_2 pressure has been presented by Calvin's group (37). In changing from high to low CO_2 concentration the insufficiency of CO_2 resulted in accumulation of RuDP which could be used as a source of glycolate. Although the chemistry of glycolate synthesis may indeed be consistent with this situation, the hypothesis cannot explain glycolate synthesis in steady state photosynthesis at low CO_2 partial pressure nor is it likely to be the determining factor for glycolate synthesis.

Oxygen concentration: The requirement of a high (20% or above) oxygen partial pressure during photosynthesis for glycolate production has been well established (5, 26, 28, 38), but not explained. In 80 or 100% O_2, though photosynthesis is inhibited, the % C^{14} incorporated into glycolate products is not reduced (N. E. Tolbert,

unpublished). Below 20% oxygen, glycolate synthesis ceases. Thus the conditions for glycolate synthesis are those which occur in nature--high O_2 and low CO_2. Glycolate oxidase, a FMN containing enzyme, in vitro has a low affinity for oxygen, so high oxygen concentration would lead to the oxidation of glycolate rather than accumulation. Thus aerobic conditions favor both glycolate synthesis, and because of the oxidase, its metabolism. Glycolate oxidase can also be coupled to DCPIP or to a naturally occurring quinone-like material which we have not identified. The nature of the oxygen requirement for glycolate production is unknown. Warburg has suggested that aerobic oxidation of a C_2 product of photosynthesis to glycolate must occur first in order that O_2 evolution can generate reducing power (5).

Effect of phosphate: The impairment of photosynthetic activity by washing algae with water is well recognized. Three washings of Chlamydomonas reinhardti reduced their photosynthetic ability 80% as measured by $C^{14}O_2$ fixation (Table 4). This inhibition was completely restored by 3×10^{-3} M phosphate, phosphoglycolate or phosphoglycerate (39). Glucose-6-P was much less effective and ATP was ineffective. However, certain other non-phosphate compounds such as serine also restored the photosynthetic activity, which is indicative of the complexity of the phenomenon.

Table 4. Effect of washing and phosphate on CO_2 fixation by Chlamydomonas.

	Total c/s in 10 min. by 1 ml aliquot	
Additions*	Unwashed algae	Washed algae
none	2,700	546
3.3×10^{-3}M phosphate	2,960	3,100
3.3×10^{-3}M phosphoglycolate	2,560	3,050

*All solutions were adjusted to pH 7.

The products of $C^{14}O_2$ fixation depend on the phosphate in the medium. Very fast labeling of glycolate occurs in algae when photosynthesis is performed in the absence of phosphate (Table 5). These algae were lightly centrifuged from their growth medium and resuspended in water. If the algae were resuspended in .001 M phosphate, no C^{14}-labeled glycolate was formed in 40 seconds and a 1 to 2 minute period was required before any glycolate-C^{14} was synthesized (G. Orth and N. E. Tolbert, unpublished). Whittingham's data on glycolate excretion confirms this phenomenon (29). However, the presence of phosphate in the medium does not prevent the algae from ultimately synthesizing and excreting large amounts

Table 5. Effect of phosphate on $C^{14}O_2$ fixation by Chlamydomonas.*

Time	No phosphate		.001 M phosphate	
	Total	Glycolate	Total	Glycolate
seconds	c/s	c/s	c/s	c/s
10	167	19		
30	464	93	96,100	0
60	972	267	147,100	440
120	4857	1965	102,100	4178

*Although fixation was much less without phosphate exact comparison between the experiments cannot be made because they were run at different times.

of glycolate after 10 to 20 minutes. Addition of phosphoglycolate to the algal medium is even more inhibitory to the rate of C^{14} labeling of glycolate than orthophosphate, although it substitutes completely for orthophosphate in stimulating total CO_2 fixation. The great need for orthophosphate during $C^{14}O_2$ fixation by isolated chloroplasts (40) can be replaced by equi-molar amounts of phosphoglycolate. However, phosphoglycolate phosphatase activity of the chloroplasts is not sufficient to have hydrolyzed much of the phosphoglycolate to orthophosphate during the course of the experiment.

Manganese: Tanner et al. (17) claimed that Mn deficient Chlorella do not produce glycolate. However, their data show that with Mn deficient cells, which did not produce glycolate, 13.0% of the C^{14} fixed in 1 hr. was present as glycine and serine while with normal cultures 9.4% of the C^{14} was present as glycolate, glycine and serine. Thus if one considers all the products of the glycolate pathway, Mn deficiency did not inhibit their synthesis, but instead altered glycolate accumulation. A similar effect was obtained by blue light. Mn^{++} will serve as cofactor for glycolate phosphatase but so will Mg^{++}.

Recapitulation

Glycolate synthesis during photosynthesis requires low CO_2 concentration (less than 0.4%), high O_2 concentration (20%) and high light intensity. The phenomenon of a specific loss, or movement or excretion of phosphoglycolate and glycolate by algae or by chloroplasts remains unresolved. Glycolate excretion occurs most rapidly with young algae cultures in the absence of phosphate buffer. Manganese deficient cultures do not excrete glycolate. Much of the fixed CO_2 of photosynthesis pass through the glycolate pathway which appears to be a metabolic sequal from the path of carbon. Plant enzymes are known for each step of this pathway and

several of the first ones in the sequal are absent in etiolate plants. Glycolate synthesis is associated with the chloroplasts and C^{14} is incorporated into its products very rapidly during photosynthesis. The glycolate is rapidly converted to sucrose in the light.

Function of Glycolate Pathway

Amino acid synthesis: The glycolate pathway does not exist solely for production of glycine and serine for protein synthesis. During $C^{14}O_2$ photosynthesis glycine and serine become labeled much more rapidly than other amino acids, and in turn they lose C^{14} equally rapidly if the $C^{14}O_2$ is exchanged for $C^{12}O_2$.

CO_2 fixation: In previous sections reasons were given why the glycolate is not thought to be an alternate pathway for CO_2 fixation. Most pertinent was phosphoglycolate production by chloroplasts in the dark from sugar phosphates of the photosynthetic carbon cycle.

Anion exchange: We advanced the concept in 1956 that glycolate excretion occurred for the purpose of anion exchange with bicarbonate (26). Since that time the requirement for low CO_2 partial pressure has been well documented for glycolate synthesis and excretion. This requirement is consistent with the bicarbonate exchange theory. At high CO_2 concentration sufficient diffusion of bicarbonate and CO_2 could occur for photosynthesis, and the need would not exist for an exchange mechanism to move bicarbonate rapidly into the chloroplasts. I do not feel that this hypothesis has been either refuted or confirmed to date.

Metabolic link between chloroplasts and cytoplasm: Since glycolate is rapidly metabolized to sucrose, and since it appears outside of chloroplasts, the glycolate pathway may constitute a link between the photosynthetic carbon cycle of the chloroplast and the synthetic pathways of the cytoplasm (1, 15, 16). Thus there exist two reservoirs of serine. One, inside the chloroplast, is carboxyl labeled and similar to photosynthetically produced phosphoglycerate, and one, outside the chloroplast or in the cytoplasm, is uniformly labeled as dictated by the glycolate pathway. Similarly there should exist two reservoirs of sucrose with similar labeling differences. An experiment to test this would be to determine the C^{14} labeling patterns of sucrose of the chloroplast in comparison with labeling in sucrose of the phloem.

The glycolate lost by the chloroplasts is converted to glycerate. The excretion of glycerate by the chloroplasts, as already noted, would not alter the basic function of a carbon transport

mechanism from the chloroplasts to the cytoplasm. The glycerate may be converted to sugars or metabolized to phosphoenol pyruvate and on to malate and aspartate. Added glycolate-C^{14} results in considerable synthesis of malate-C^{14} and when the glycolate pathway in tobacco leaves was blocked by the α-hydroxysulfonate inhibitors, C^{14} labeling of a large isocitrate reservoir was completely blocked (4). These findings suggest that further search for glyoxylate cycle enzymes for isocitrate synthesis in tobacco leaves is merited.

Permease system: The excretion of glycolate by algae or by chloroplasts has the characteristics of a permease system in which the action of phosphoglycolate phosphatase would provide direction. This theory requires that phosphoglycolate but not glycolate might cross the membrane. Phosphoglycolate is observed to come out of the chloroplasts. Glycolate-C^{14} is not absorbed by chloroplasts at significant rates (16). A slow, light-stimulated, oxidation of it to CO_2 has been reported (41). Moses and Calvin (42) extended this theory when they reported that glycolate contains a major portion of the tritium incorporated from T_2O during photosynthesis. They suggested that the glycolate pathway serves to carry not only carbon but hydrogen from the photosynthetic apparatus. (However, clarification of their results are needed since the apparent high tritium content of glycolate could have resulted from glycolate sublimation from the paper chromatogram onto the film.) Such a system would be analogous to the role of the glycerol phosphate dehydrogenases in transport of reducing power into the mitochondria. Glycolate would be oxidized in the cytoplasm to glyoxylate. Glyoxylate, unlike glycolate, can reenter the chloroplasts (16) where there is a NADPH linked glycolate reductase to reduce it back to glycolate with photosynthetic assimilatory power. A major fault of this scheme or any other scheme involving a combination of glycolate oxidase and glyoxylate reductase is that glycolate oxidase has not been coupled to oxidative phosphorylation (32) or NAD reduction. If phosphoglycolate were synthesized from phosphoglycolaldehyde, as mentioned previously, the diose phosphate dehydrogenase system would create sufficient assimilatory power for the resynthesis of sugars from glycolate.

Glycolate metabolism and O_2 evolution: Several similarities exist between O_2 evolution and glycolate accumulation or excretion during photosynthesis which taken all together suggest that they may be related. Both processes are inhibited by Mn deficiency and by CMU. Glycolate oxidase contains FMN and it will reduce DCPIP. Both these factors have been associated with the Hill reaction. Homann and Gaffron (43) have indicated that CMU changes

the reactivity of excited FMN. Glycolate inhibition of the Hill reaction when DCPIP is the oxidant (44), could be due to inhibition by the $DCPIPH_2$ (45) produced from glycolate oxidation. High O_2 inhibits photosynthesis, but glycolate synthesis is favored by high O_2 pressure. Roux's data indicates that orthophosphate or a phosphorylated compound may be an intermediate for O_2 in photosynthesis (46). The unexplained suppression of rapid glycolate labeling by phosphate or phosphoglycolate may be related to some such phosphate requirement. Glycolate synthesis and metabolism only during photosynthesis in a high O_2 atmosphere, suggest that its metabolism may be necessary to maintain in a reduced state some auto oxidizable component of the chloroplast.

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AN EVALUATION OF THE CARBON REDUCTION PATHWAYS OF PHOTOSYNTHESIS

Martin Gibbs

In general the term photosynthetic carbon reduction pathway, reductive pentose-P pathway or Calvin cycle is conventially used to describe the cyclic reaction sequence involved in the conversion of CO_2 to carbohydrate in a chlorophyll-containing cell. Carbohydrate is considered here to be D-fructose-6-P.

The historical development of the cycle has been ably described by Bassham and Calvin[1]. The cycle as it is usually presented was initially set down in paper XXI of the classic series of papers deriving from Calvin's laboratory[2]. The essential points of the cycle are:

1. conversion of D-ribulose-5-P to D-ribulose-1,5-diP by ATP in the presence of phosphoribulokinase.
2. carboxylation of ribulose-1,5-diP to produce a yet-unidentified 6-carbon intermediate which is hydrolytically cleaved to produce to 2 molecules of D-3-P-glycerate catalyzed by ribulose-1,5-diP carboxylase.
3. phosphorylation of 3-P-glycerate and subsequent reduction by 1,3-diPGA by reduced pyridine nucleotide catalyzed by PGA kinase and glyceraldehyde-3-P dehydrogenase, respectively.
4. conversion of D-glyceraldehyde-3-P to fructose-6-P catalyzed by triose-P isomerase, D-fructose-1,6-diP aldolase, and fructose-1,6-diphosphatase.
5. subsequent steps catalyzed by transketolase, aldolase, sedoheptulose-1,7-diphosphatase, and xylulose-5-P-3-epimerase accomplish the conversion of fructose-6-P to ribulose-5-P.

The stoichiometry of the cycle may be formulated in terms of conversion of CO_2 and [H], in the form of ATP and reduced pyridine nucleotide, to fructose-6-P in the following way. $6\ CO_2 + 18\ ATP + 10\ H_2O + 12\ PNH \longrightarrow$ fructose-6-P + 18 ADP + 12 PN^- + 17 Pi. Thus, for the entrance of 6 molecules of CO_2 into the cycle, 6 molecules of ribulose-1,5-diP are required. These give rise to 12 molecules of 3-P-glycerate which is converted to glyceraldehyde-3-P and its equivalent, dihydroxyacetone-P of the 12 molecules of triose-P so formed, 10 are needed to complete 2 turns

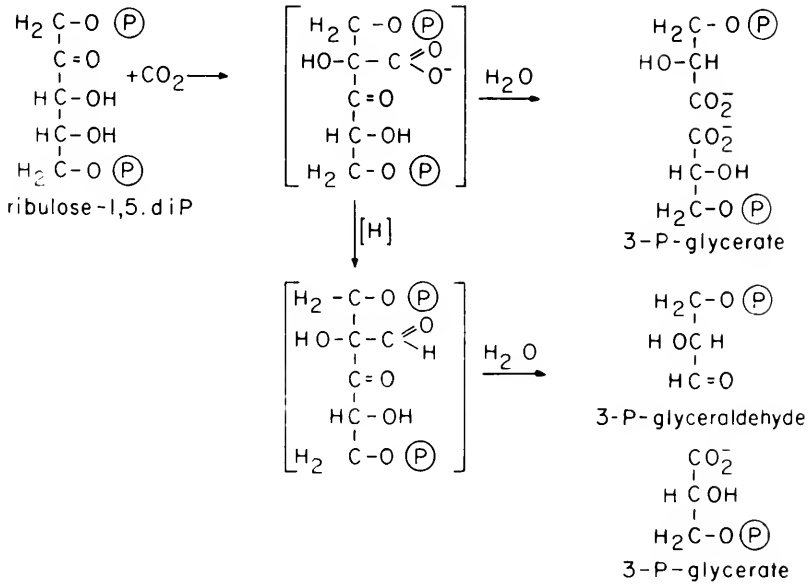
of the cycle, and 2 molecules can be utilized for fructose-6-P synthesis.

Evidence for this carbon reduction cycle from CO_2 to fructose-6-P was based on: (a) kinetic tracer studies of *in vivo* systems (b) presence of enzymes catalyzing the postulated reactions (c) distribution of C^{14} in the various intermediates following assimilation of $C^{14}O_2$, and (d) information obtained with inhibitors.

Kinetic data

Evidence for the functional role of this cycle is derived mainly from kinetic studies with $C^{14}O_2$ carried out by Calvin, Benson, Bassham and associates[1]. Exposure of photosynthesizing algae to $C^{14}O_2$ gave rise to most of the isotope in 3-PGA, labeled mainly in the carboxyl-carbon. The cyclic nature of cycle was revealed by the appearance of isotope in the α and β positions of 3-PGA. The discovery of rapidly labeled D-sedoheptulose-7-P and D-ribulose-1,5-diP led to their inclusion. The reciprocal changes in reservoir sizes of ribulose-1,5-diP and 3-PGA observed when algae were subjected to light and dark periods and to changing partial pressures of CO_2 indicated a close relationship between these 3 compounds[3]. While reinvestigating the kinetics of appearance of C^{14} in components of the reductive carbon cycle in *Chlorella* photosynthesizing under steady-state conditions. Bassham and Kirk[4] could account for only one molecule of 3-PGA for each CO_2 taken up. This observation contrasts with the formation of 2 molecules of 3-PGA per carboxylation when the light is turned off. They postulated that light and the intact cell carry out a reductive carboxylation while in the *in vitro* system or in the dark carboxylation followed by hydrolysis occurs. Both of these mechanisms propose hydrolysis of the hypothetical 6-carbon ketoacid prior to reduction. An alternative mechanism is outlined in figure 1. Here, reduction of the ketoacid to a keto-aldehyde prior to hydrolysis would result in the formation of one molecule of PGA and one of glyceroldehyde-3-P.

Reductive Carboxylation of Ribulose Diphosphate



Another explanation to account for the unexpected finding of Bassham and Kirk was recently proposed by Bassham[5]. Using Lynen's[6] system for the biosynthesis of fatty acids as a model, he proposed that the enzymes of the carbon cycle are highly organized as a multifunctional enzyme system. He has also speculated that sulfhydryl groups and thiamine pyrophosphate would form the bridge between the enzymes and substrates. The importance of an enzyme disulfide functioning in photosynthesis was suggested by Gibbs and Calo[7] who observed that arsenite did not inhibit the photochemical act (formation of ATP, TPNH and O_2) or any of the enzymes usually associated with the Calvin cycle. This ineffectiveness toward the broken system contrasts sharply with a 90% inhibition of CO_2 fixation by the intact chloroplast in the presence of 1×10^{-5}M arsenite.

The observation of Bassham that the ribulose-1,5-diP carboxylase system may act differently in the dark or *in vitro* as contrasted to *in vivo* in the light indicates that the primary carboxylation reaction needs reinvestigation.

Enzyme data

Enzymes of the reductive pentose-P cycle are widespread in nature. None of them with the possible exception of the TPN-linked glyceraldehyde-3-P dehydrogenase[8] appear to be the exclusive property of the photosynthetic cell. In contrast to studies with glycolysis, the urea cycle and pyrimidine synthesis where the least active enzyme had a capacity several-fold higher than the overall system, a comparison of the activities of the individual enzymes of reductive pentose-P cycle with the rate of photosynthetic CO_2 fixation of intact cells revealed certain deficiencies. Peterkofsky and Racker[9] reported low activities for transaldolase, ribulose-1,5-diP carboxylase, sedoheptulose-1,7-diphosphatase as well as for fructose-1,6-diphosphatase. Richter[10] and Fewson *et al.*[11] reported the absence of fructose-1,6-diP aldolase from extracts of blue-green algae. Szymona and Doudoroff[12] and later Richter reported only small amounts of this aldolase in extracts of *Rhodopseudomonas spheroides*. While caution must be exercised in evaluation of these observations, it does suggest that a search should be made for alternative enzymes catalyzing individual steps of the cycle, particularly where apparent enzyme deficiencies are observed.

Isotope distribution data

The earliest experiments of Calvin and coworkers[1] showed that brief exposure of photosynthesizing higher plants and algae to $C^{14}O_2$ produced 3-PGA labeled mainly in the carboxyl carbon and

a hexose sugar labeled predominantly in carbon atoms 3, 4 (C-3, C-4). Subsequently isotope entered the α and β carbons of 3-PGA and C-1, C-2, C-5, C-6 of the hexose. Similarity in distribution of C^{14} between both halves of hexose provided evidence that the conversion of 3-PGA to hexose occurred via the reversal of the Embden-Meyerhof pathway. Using the Leuconostoc mesenteroides technique, Gibbs and Kandler[13] reported that glucose isolated from starch and sucrose produced during short periods of photosynthesis possessed an asymmetric distribution of C^{14} rather than the predicted symmetrical distribution. Many explanations such as transaldolase exchange reactions and pool dilution of the dihydroxyacetone-P have been offered to circumvent this unexpected finding.

The absence of aldolase in extracts of blue-green algae and the report of Kandler[14] that the distribution of C^{14} in the products formed during photosynthesis in $C^{14}O_2$ by Anacystis nidulans was similar to those obtained with Chlorella, an alga which contains aldolase, prompted an examination of the distribution pattern of C^{14} in A. nidulans polysaccharide.

Table 1. DISTRIBUTION OF C-14 IN POLYSACCHARIDE GLUCOSE FORMED DURING PHOTOSYNTHESIS USING A. nidulans and C. pyrenoidosa

Time (sec)	C-1	C-2	C-3	C-4	C-5	C-6
			Chlorella pyrenoidosa			
5	5.0	5.4	73	100	0.8	0.8
10	5.2	5.4	80	100	1.5	2.4
20	14	12	86	100	8.5	9.6
30	20	17	86	100	14	18
			Anacystis nidulans			
5	5.6	1.6	55	100	0.9	0.9
10	9.3	5.5	68	100	0.8	2.1
20	18	8.0	77	100	3.9	9.0
30	26	21	73	100	11	19

Relative specific activity of the carbons of glucose on the basis of C-4=100

Table I shows the asymmetric pattern of distribution of C^{14}

in the glucose of the polysaccharide. These results and those of Kandler and Richter suggest the following: (1) aldolase is present in Anacystis and Chlorella and the reductive carbon cycle functions in both organisms but the enzyme is not detected in extracts of Anacystis for various reasons; (2) aldolase is not involved with the conversion of CO_2 to carbohydrate during photosynthesis; (3) different mechanisms of photosynthetic assimilation of CO_2 operate in the 2 algae but both mechanisms give rise to the same distribution of C^{14} pattern.

Feeding of labeled acetate and CO_2 in the dark

It became of interest to determine whether asymmetry of label in the glucose was a light-dependent event. It was found that $C^{14}O_2$ and acetate-1-C^{14} given to Chlorella produced an asymmetrically labeled glucose; in contrast, acetate-2-C^{14} formed a symmetrically labeled hexose (Table 2).

Table 2. DARK INCORPORATION OF $C^{14}O_2$, ACETATE-1-C^{14}, AND ACETATE-2-C^{14} INTO GLUCOSE OF C. pyrenoidosa

Subst.	Time (hour)	C-1	C-2	C-3	C-4	C-5	C-6
$C^{*4}O_2$	1	17	14	61	100	4	4
acetate-1-C^{14}	1	40	35	89	100	23	26
acetate-2-C^{14}	0.5	100	103	55	50	103	110
acetate-2-C^{14}	1	100	94	50	53	105	104

Light is apparently not necessary for the formation of asymmetrically labeled glucose from CO_2. In the dark, the labeled acetates are apparently incorporated into glucose by 2 super-imposed over-all mechanisms; a rapid one involving the citric acid cycle and glycolytic pathway yielding symmetrically labeled glucose and a slower operating carbon reduction cycle which causes asymmetry. The conclusion can be drawn that asymmetry is a property of the carbon reduction cycle and not of the pathways leading to carbohydrate involving the reversal of respiration[15].

Feeding of labeled sugars

To test whether asymmetry is characteristic of the reductive carbon cycle or of respiratory pathways, specifically labeled glucose and ribose were fed to algae. Further information of carbohydrate metabolism was obtained by using Chlorella pyrenoidosa[16] which contains aldolase and Tolypothrix tenuis which is lacking aldolase.

Table 3. PERCENT DISTRIBUTION OF C^{14} IN GLUCOSE POLYSACCHARIDE AFTER FEEDING OF LABELED SUGARS TO C. pyrenoidosa and T. tenuis

Substrate	Organism	C-1	C-2	C-3	C-4	C-5	C-6
glucose-1-C^{14}	Chlorella	83	2	2	2	0	11
glucose-2-C^{14}	Chlorella	3	72	5	2	16	2
glucose-6-C^{14}	Chlorella	17	3	1	8	0	71
glucose-1-C^{14}	Tolypothrix	80	3	11	3	1	2
glucose-2-C^{14}	Tolypothrix	25	56	13	1	2	3
glucose-6-C^{14}	Tolypothrix	10	2	2	1	0	87
ribose-1-C^{14}	Tolypothrix	41	15	28	2	2	2

After 2 hours of incubation with the labeled substrates, it is evident that Chlorella which contains aldolase converts glucose to triose phosphates and back to polysaccharide via the Embden-Meyerhof pathway. The distribution of isotope suggest that the blue-green alga which does not contain aldolase possesses an extremely active pentose-P cycle. Limited equilibration between the two halves of the glucose molecule in Tolypothrix in contrast to Chlorella is taken as further evidence of a lack of aldolase in the blue-green cell. On the other hand, an extremely rapid pentose-P cycle together with an active glyceradehyde-3-P oxidizing system could prevent the reformation of fructose-1,6-diP from glyceraldehyde-3-P. Structural differences between the two kind of algae may effect the handling of the labeled sugars. The Chlorophyceae algae possess a definite chloroplast and mitochondria. In contrast, the Myxophyceae are the only algae in which the pigments are not centered in definite chromatophores and in which typical mitochondria type structures are not evident. However, enzymes of the pentose-P cycle and Embden-Meyerhof pathways are not usually associated with cellular particulate matter.

Studies with isolated chloroplasts

The pioneering work of Arnon and his associates indicated that the isolated chloroplast might afford a useful technique in elucidating the nature of asymmetry in the reductive pentose-P cycle. Arnon et al.[17] had provided evidence that both the intact chloroplast and reconstituted chloroplast system had a complete and functioning cycle. This conclusion was based essentially on the appearance of C^{14} in some compounds of the cycle after light-induced $C^{14}O_2$ uptake. Since the C^{14} can spread to a number of compounds by a series of reactions which do not necessarily constitute a cycle, its appearance in a few compounds of the reductive pentose-P cycle is only suggestive evidence that a

functioning reductive CO_2 cycle is present in the isolated systems. The conclusion of Arnon[2] was tested by supplying intact spinach chloroplasts and reconstituted spinach and pea chloroplast preparations with $C^{14}O_2$, light, and PPNR[18]. In addition to $C^{14}O_2$, broken spinach chloroplasts were supplied with D-ribose-5-P or ribulose-1,5-diP. With the pea material prepared by the method of Stocking, glycerate-3-P-1-C^{14} replaced the $C^{14}O_2$ and pentose-P[19]. Similar experiments have been reported by Trebst and Fiedler[19].

Table 4. DISTRIBUTION OF C^{14} IN GLUCOSE-6-P AND GLYCERATE-3-P FORMED DURING $C^{14}O_2$ FIXATION BY INTACT SPINACH CHLOROPLASTS

Carbons of glucose-6-P	4 min.	10 min.	40 min.
C-1	74	79	92
C-2	73	75	82
C-3	97	86	94
C-4	100	100	100
C-5	--	61	92
C-6	51	62	--
Carbons of 3-PGA			
COOH	100	100	100
CH_2OH	55	63	75
CH_2OP	62	63	80

Carbon content is based on C-4 or COOH=100

The most striking results of these data (Tables 4 and 5) was the rapid spread of tracer which had taken place in the compounds derived from the whole chloroplast experiments. In sharp contrast, the spread of C^{14} was low in those compounds isolated from the reconstituted systems. It is of significance that this difference could not have been predicted on the basis of detecting which compounds were labeled and which were unlabeled since C^{14} appeared in essentially the same compounds in the experiments with both whole chloroplasts and reconstituted systems. It is concluded from these data that the intact chloroplasts possess a complete reductive pentose-P cycle even though the rate of CO_2 assimilation was only 2% of that reported for intact photosynthetic tissue. This cycle was operating at a limited rate, if at all, in the broken preparations. This inability of the reconstituted system to carry out a complete cycle indicates that the present methods of preparing reconstituted chloroplast systems must be modified. Perhaps this failure should have been expected since the intact chloroplasts were able to reduce CO_2 at a small

Table 5. DISTRIBUTION OF C^{14} IN GLUCOSE-6-P, FRUCTOSE-1,6-diP, AND GLYCERATE-3-P ISOLATED FROM RUPTURED PEA CHLOROPLAST SYSTEM AFTER 40 MINUTES OF INCUBATION

	C^{14} content with substrate:	
Carbons of glucose-6-P	ribose-5-P	glycerate-3-P-1-C^{14}
C-1	4	10
C-2	4	4
C-3	60	83
C-4	100	100
C-5	1	1
C-6	1	1
Carbons of fructose-1,6-diP		
C-1	6	3
C-2	9	3
C-3	97	96
C-4	100	100
C-5	3	4
C-6	3	5
Carbons of 3-PGA		
COOH	100	100
CHOH	1	7
CH_2OP	2	8

Carbon content is based on C-4 or COOH=100

rate when compared to the intact cell. All attempts to increase the basic rate of CO_2 reduction by the intact higher plant chloroplast in this laboratory and apparently in others have resulted in uniform failure. It would appear that the present methods of preparing chloroplasts, 0.35 M NaCl, 0.4 M sucrose, or density gradient, are not suitable for elucidation of the path of carbon. Even though isotope spreads rapidly throughout the carbon atoms of the hexose in the intact chloroplast, the low overall rate is still an important problem that cannot be ignored. The data obtained with these preparations substantiate the observation of Bassham and Kirk[4] that differences may occur between the intact and fragmented systems. Integrity of a multifunctional enzyme system may be a key factor in elucidation of the cycle.

Inhibitor data

The site of iodoacetamide inhibition has been reported to phosphoribulokinase[20] while the site of arsenite inhibition cannot be assigned at present[7].

D-threose-2,4-diP has been reported[21,22] to specifically

inhibit muscle and yeast glyceraldehyde-3-P dehydrogenase. Park et al.[23] observed that threose-2,4-diP inhibited CO_2 fixation by sonically ruptured spinach chloroplasts and assigned the site of inhibition as the TPN-linked triose-P dehydrogenase. Gibbs and Bamberger[24] have repeated these experiments using the intact spinach chloroplasts. Threose-2,4-diP (10^{-4} M) did not affect CO_2 assimilation. The diphosphate may not penetrate the chloroplast but evidence obtained in this laboratory shows that other diphosphates, sedoheptulose and fructose, can affect CO_2 fixation. Indeed, when threose-2,4-diP (1.1×10^{-4} M) was incubated with the rabbit muscle, DPN-spinach or TPN-spinach triose-P dehydrogenase, the mammalian and the DPN-linked plant enzymes were inhibited completely while the TPN-enzyme was unaffected. These data indicate that the DPN-dehydrogenase is not a rate-limiting step in the chloroplast carbon cycle. Threose-2,4-diP would appear to be of little value in elucidating the enzymes involved in the carbon reduction cycle in the intact chloroplast.

The inhibition of the enzyme is apparently due to the similarity between threose-2,4-diP and 1,3-diPGA molecules. The data obtained here suggest that the mechanism of the TPN-enzyme may differ from that of its nucleotide counterpart. Perhaps, 1,3-diPGA is not the real substrate for this enzyme and therefore, inhibition does not occur.

An unexpected finding was the inhibition by sedcheptulose-7-P of CO_2 fixation by the intact chloroplast. In contrast to threose-2,4-diP, sedoheptulose-7-P inhibited the rabbit muscle enzyme as well as both triose-P dehydrogenases from spinach chloroplasts (Table 6).

Table 6. EFFECT OF D-SEDOHEPTULOSE-7-P ON GLYCERALDEHYDE-3-P DEHYDROGENASE FROM RABBIT MUSCLE AND SPINACH CHLOROPLAST

Enzyme source	S-7-P	Back reaction	Forward reaction
		% inhibition	
muscle-DPN	5×10^{-4} M	14	28
muscle-DPN	1×10^{-3} M	37	--
muscle-DPN	2×10^{-3} M	53	--
chloroplast-DPN	5×10^{-4} M	33	33
chloroplast-DPN	1×10^{-3} M	66	--
chloroplast-TPN	5×10^{-4} M	10	40
chloroplast-TPN	1×10^{-3} M	50	--

Fructose-6-P, D-erythrose-4-P, D-glucose-6-P, glycolaldehyde-P and D-ribose-5-P had no inhibitory effects on any of the dehydrogenases.

Sedoheptulose-7-P may act as a controlling agent of the carbon reduction cycle. Accumulation of this compound could effectively block the cycle in the reconstituted systems. Inorganic phosphate was suggested earlier to have a similar function[25].

Conclusion

The reductive pentose-P scheme as postulated by Calvin and his associates can account for most *in vivo* and *in vitro* observations summarized here. While the role of asymmetry in the labeling pattern is not unique to autotrophic systems, its significance is still unknown in the photosynthetic process. The apparent absence of fructose-1,6-diP aldolase from extracts of the Cyanophyceae poses a problem. Either the correct conditions for detecting this enzyme have not been employed or the more primitive photosynthetic cell may have a reductive carbon cycle which differs from the higher plants. A perplexing problem is the low rate of CO_2 fixation by the isolated higher plant chloroplasts, especially, when the same preparations reduce TPN with concomitant ATP formation to satisfy the demands of intact cell photosynthesis. It may be that *in vitro* preparations presently used may or may not duplicate the carbon cycle of the *in vivo* process. There are no reports on the isolation of particles from unicellular material capable of assimilating CO_2 at a rate equivalent to or higher than that reported for the higher plant chloroplast. The photoelectron transport system is apparently so intimately connected with the carbon reduction cycle that physical separation may not be possible. Perhaps the connecting link between the two systems is more than ATP and TPNH.

ACKNOWLEDGMENTS

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THE PRODUCTION OF GLYCOLLATE DURING PHOTOSYNTHESIS

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A direct investigation of the compounds first formed during photosynthesis became possible only with the exploitation by M. Calvin of the radioactive isotope of carbon, carbon 14, coupled with the technique of paper chromatography and led to formulation of the photosynthetic carbon reduction cycle[1].

Most of the investigations by Calvin and his colleagues were of photosynthesis at concentrations of carbon dioxide far higher than that in normal sir. In our laboratory we have investigated the products of photosynthesis in Chlorella over a range of concentrations of CO_2 above and below that in air. We have confirmed[2] previous observations[3,4] that at low carbon dioxide concentrations a large fraction of the carbon dioxide fixed in photosynthesis is converted to glycolic acid, and to the amino acids glycine and serine. As the carbon dioxide concentration is increased production of glycollic acid and glycine decreases and sucrose becomes the major product. Other compounds, e.g., alanine, malic and aspartic acids, are not markedly affected by changing the carbon dioxide concentration within the range studied by us and represent a relatively small fraction of the total carbon fixed.

EXPERIMENTAL METHODS

In all the experiments three- to four-day-old cultures of Chlorella pyrenoidosa (Emerson Strain) grown on 4% carbon dioxide in air, at constant light intensity and temperature (22° C), were used. Cells were harvested by centrifugation, washed once with distilled water, and resuspended in potassium dihydrogen phosphate solution (10^{-4} M; pH 4.5-5.0). A cell density of 1650 ul wet packed volume of cells per 100 ml of solution was used.

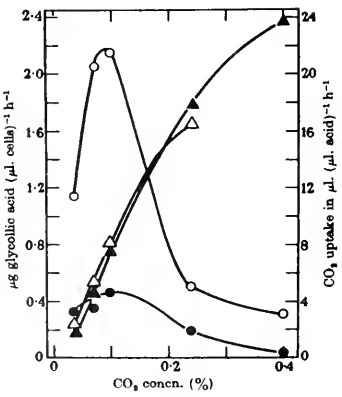
The cell suspension was illuminated in a perspex chamber 5 mm thick and 19 cm in diameter, the incoming gas system aerating the suspension through a finely perforated polythene tube. Carbon dioxide uptake during photosynthesis was measured by passing the outflowing gas through an infrared CO_2 analyser. Glycollic acid in the medium was estimated by the colorimetric method of Calkins[5], after centrifuging the cells (3000 x g for 5 min) and concentrating the supernatant in vacuo at 30° C. A slight brownish color was given by samples taken after the cells had been aerated in the dark for 30 min, and this reading was subtracted from that of the bright purple obtained after 30 min subsequent photosynthesis. Isoniazid gave no colour with the glycollic acid reagent.

The products of photosynthesis when Chlorella was fed $^{14}CO_2$ were determined using the techniques developed by Calvin, et al.[1]. The suspension was first aerated in the dark for 30 min and then illuminated at 20,000 lux for a further 30 min, after which the non radioactive CO_2 was replaced with $^{14}CO_2$ at the same flow rate and total CO_2 concentration. Samples were taken directly into boiling ethanol. After centrifugation, the cell residues were reextracted with

20% ethanol and the two extracts combined. Subsequent treatment followed the procedure of Bassham and Calvin[1]. Radioactivity in the compounds located by radioautography was counted directly on the paper chromatograms using a thin end-window Geiger-Muller tube.

PHOTOSYNTHETIC GLYCOLLATE PRODUCTION

The production of glycollate was first determined during photosynthesis at a high light intensity (56,000 lux) and with different concentrations of CO_2. These concentrations of CO_2 are those in the gas phase bubbling through the suspension; the effective CO_2 concentration at the cell surface was estimated to be of the order of one-tenth of this. The results are shown in Fig. 1. The maximum rate of glycollate excretion was reached at a CO_2 concentration in the gas phase of 0.1%; above this value the rate declined rapidly, reaching a very low value at 0.4% CO_2. The rate of photosynthesis increased almost linearly over the range of 0.03 to 0.24% CO_2. Above 0.24% the rate increased less with increase in concentration, but saturation was not reached with the highest concentration used, i.e., 0.4%. At higher CO_2 concentrations preliminary experiments show that as photosynthesis becomes CO_2 saturated the excretion of glycollate remains low. A time course of $^{14}CO_2$ incorporation into individual ethanol soluble compounds during photosynthesis at 0.1% (="low CO_2") the point of maximum glycollate excretion, and 0.4% CO_2 (="high CO_2") is shown in Fig. 2. At "low CO_2" there is more activity in glycollate and glycine despite a lower rate of CO_2 fixation. At "high CO_2", activity in the two carbon compounds is suppressed while that in PGA and sucrose increased sharply.



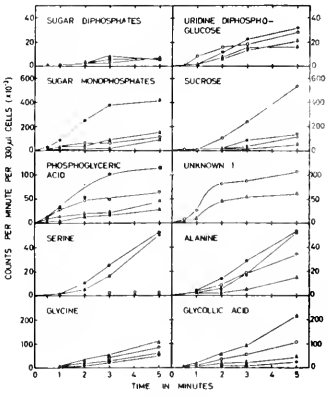


Fig. 1. The effect of carbon dioxide concentration on glycollic acid excretion and the rate of carbon dioxide uptake in the presence and absence of isoniazid. In the absence of isoniazid: ●, glycollic acid excretion; ▲, carbon dioxide uptake. In the

presence of isoniazid: ○, glycollic acid excretion; △, carbon dioxide uptake.

Fig. 2. The time course of ^{14}C incorporation into individual compounds of the ethanol-soluble fraction.

high carbon dioxide concentration
low carbon dioxide concentration — in the absence of isoniazid

high carbon dioxide concentration
low carbon dioxide concentration — in the presence of isoniazid

We have found [2,6] that the drug isonicotinyl hydrazide has a marked effect on the products of but not the rate of photosynthesis in Chlorella. Its use results in a 250% increase in glycollate production for the same CO_2 uptake (Table I). $^{14}CO_2$ studies show that an equal amount of glycine is produced (Fig. 2), and if both carbon atoms of glycollate and glycine are supplied by the CO_2 they would together equal 1/2 of the total CO_2 uptake. This accumulation of 2 carbon compounds in the presence of INH results from blocking the conversion of glycine to serine and subsequent products, e.g., alanine, aspartate and malate (see Figs. 2 and 3).

Table I. Effect of isoniazid concentration on the rate of photosynthesis and glycollate excretion.

Isoniazid concentration	Rate of photosynthesis ul CO_2/ul cells/hr	Rate of glycollate excretion ug/glycollate/ul cells/hr
0	2.77	0.435
10^{-4} M	2.62	0.772
10^{-3} M	3.42	0.830
10^{-2} M	3.13	1.26

It is believed that (at low CO_2) serine is converted to aspartate and malate via phosphoenol pyruvate for the following reasons: inhibition by INH of $^{14}CO_2$ incorporation into malate and aspartate can be observed within 5 sec of exposure to $^{14}CO_2$ and before glycollate and glycine are active. This activity in aspartate and malate is largely confined to the C_4 position in the controls, but on the other hand INH does not inhibit dark $^{14}CO_2$ fixation into aspartate and malate, incorporation of the activity from ^{14}C glucose into aspartate and malate, or PEP carboxylase in vitro.

The derivation of the carbon skeletons of glycollate and glycine from intermediates of the carbon reduction cycle was shown by an experiment in which the ethanol soluble compounds of Chlorella were labelled during photosynthesis in 0.1% CO_2 containing $^{14}CO_2$. After 5 min the $^{14}CO_2$ was replaced by $^{12}CO_2$, and the subsequent changes in the intermediates are shown in Fig. 3.

The intermediates may be divided into 3 classes: 1) substances in which the radioactivity decreased rapidly, e.g., PGA, sugar monophosphates and diphosphates; 2) substances showing initially a slight increase or steady level of ^{14}C subsequent to the change and then decreasing. This group contains all the amino acids; 3) substances which continued to increase in ^{14}C content, e.g., sucrose and glycollate, the latter being excreted into the medium.

The change in concentration of various intermediates in a period of darkness immediately following photosynthesis was also investigated. When the light was turned off the intermediates of the photosynthesis cycle were converted to PGA

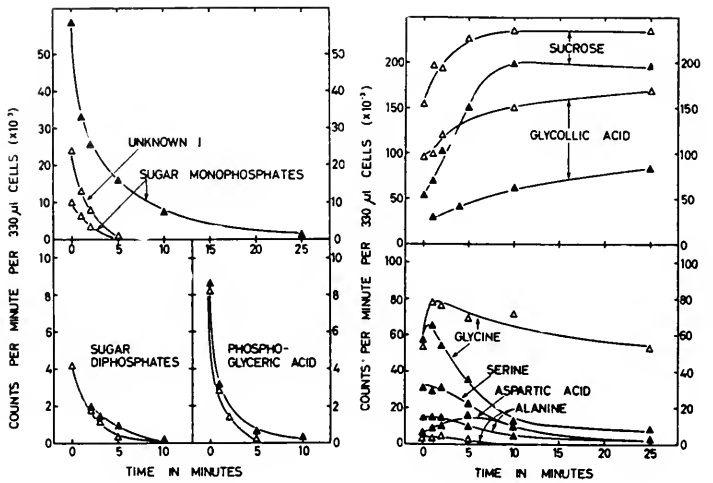


Fig. 3. The time course of loss of radioactivity in individual compounds when ^{14}C is removed from the gas phase.
▲ in the absence of INH, △ in the presence of INH.

and thence transformed to the amino acids alanine and glutamic acid. The sum total of radioactivity in serine together with glycine did not change after the illumination ceased (Fig. 4). This indicates that (in the absence of NH_4^+) at low CO_2 tensions glycine and serine are the main amino acids formed from the photosynthetic cycle, whereas in the dark immediately following the light the main cycle exit is to alanine and glutamate (the latter through the tricarboxylic acid cycle).

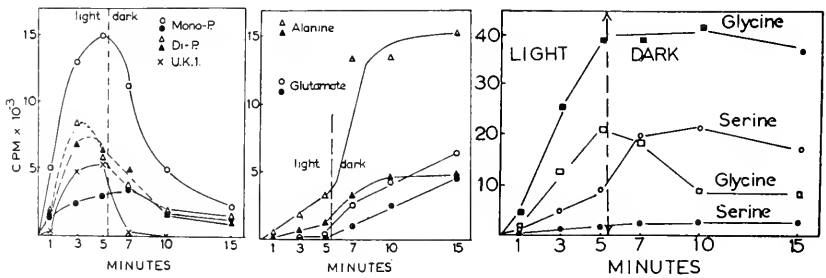


Fig. 4. The changes in time course in radioactivity upon darkening after 5 min. illumination at 0.2% CO_2. Solid symbols, +INH; open symbols, Control.

We have also shown[7] that it is these compounds that are formed when radioactive glucose is fed to Chlorella in the dark. Initially most of the radioactivity from glucose appeared in sugar mono- and diphosphates and subsequently in PGA and alanine, which lost activity after 15 min to aspartate and glutamate

In unpublished experiments we have found that the addition of ATP and ribose-5-phosphate to Chlorella cell brei in the dark results in $^{14}CO_2$ fixation into PGA, phosphoenol pyruvate, alanine, aspartate and glutamate. In both the above experiments negligible amounts of activity were found in glycollate, glycine and serine. INH did not affect these results, confirming that there is no major flow of carbon through these compounds in the dark as there is in the light.

PHOTOMETABOLISM OF GLUCOSE

a) Effect of partial pressures of carbon dioxide

The distribution of activity in the various intermediates following addition to Chlorella of a constant amount of uniformly labelled ^{14}C glucose is summarized in Table II. The results show: 1) a progressively slower utilisation of glucose at the higher concentration of CO_2; 2) a faster movement of radioactivity through the photosynthetic cycle intermediates (the sugar diphosphates and a large part of the sugar monophosphates) suggesting that the photosynthetic cycle rotates at a higher speed at higher concentrations of CO_2; and 3) that as the cycle intermediates lose their activity with time, it appears in two sets of compounds. At low concentrations of CO_2 the decrease is largely accounted for by the increase in 2C compounds and their derivatives. (We regard aspartate, malate and alanine as largely derived from 2C compounds, at least at the lower CO_2 tensions, since their formation is inhibited by INH--see below.) At higher CO_2 concentrations this formation of 2C compounds is decreased, and most of the activity lost from the cycle forms sucrose. Dependence of sucrose formation on CO_2 tension would not be expected if there was a direct conversion of glucose to sucrose, and this suggests the latter has been formed via the carbon reduction cycle.

Table II. Effect of partial pressure of CO_2 on photoassimilation of glucose. All figures are approximations based on counts applied to the chromatograms minus the counted values for the unused glucose.

		% Distribution of activity in ethanol soluble compounds					
% CO_2	Time (min)	Glycol-late	Glycollate + glycine + serine	Aspartate + malate + alanine	Sucrose	Cycle intermediates (sugar monoP, RuDP, FDP & UDPG)	% glucose unused
0	4	8.3	8.3	13.3	2.5	60	33
	11	8.3	30.0	20.0	2.7	20	0
0.01	3	8.8	8.8	9.0	5.4	61	40
	15	18.6	27.0	10.0	19.2	15	0
0.034	3	5.2	5.2	3.0	55.2	37	42
	15	8.9	16.6	3.6	72.4	6.6	0
0.09	3	9.0	9.0	3.0	57.4	23	55
	10	11.4	11.4	2.0	83.6	7	30

b) Effect of O_2 partial pressure on the photometabolism of glucose

Previous workers[8,9,10] have shown that photosynthetic glycollate production by Chlorella is increased by partial pressures of O_2 greater than that in

air. We have confirmed this in unpublished work and also investigated the effect of increasing the O_2 partial pressure on the photometabolism of glucose.

Table III compares the effect of either 20% or 99.97 O_2 on the photometabolism of glucose by Chlorella in the presence of 0.03% CO_2. Increasing the O_2 partial pressure greatly increases the activity in glycollate with a corresponding decrease in that of sucrose, suggesting that an intermediate on the normal pathway from glucose to sucrose can be oxidised to glycollate. In other experiments increasing the partial pressure of O_2 also resulted in a decreased incorporation of glucose into ethanol insoluble compounds, perhaps as a direct effect of O_2 on the formation of polysaccharides from sugar monoP. In the dark there is negligible 2C compound production from glucose-^{14}C even in the presence of oxygen or oxygen and INH.

Table III. Effect of oxygen partial pressure on photoassimilation of glucose.

Compound	Time			
	3 min		15 min	
	0.03% CO_2 in air	0.03% CO_2 in 99.97% O_2	0.03% CO_2 in air	0.03% CO_2 in 99.97% O_2
	cpm	cpm	cpm	cpm
Residual glucose glycine & serine	7254	8476	1108	666
Glycollate	498	5232	1252	11,516
Sucrose	5118	408	10,284	2786
RuDP & F 1.6 DP	490	436	228	40
UDPG	316	928	278	252
Sugar monophosphates	2628	3144	436	258
Malate & aspartate & alanine	270	912	504	384
Totals	16,574	19,536	14,090	15,902

c) Effect of isoniazid

During photosynthesis at low CO_2 tensions INH inhibits the conversion of glycine to serine, leading to an accumulation of 2-carbon compounds[2,6]. Table IV shows that INH has a similar effect on the 2-carbon compounds formed during the photometabolism of glucose at CO_2 tensions of 0.03% or less. In CO_2 free air more than 70% of the total ^{14}C in the ethanol soluble fraction was recovered in glycine and glycollate in the presence of the inhibitor. This accumulation of 2-carbon compounds is accompanied by a marked decrease in activity in alanine, aspartate and malate. The effect of INH on the incorporation of activity into sucrose is variable, but there is a marked inhibition in the presence of O_2 and we have found a similar inhibition by INH of sucrose labelled with $^{14}CO_2$ in the presence of 99.97% O_2.

Table IV. Effect of isonicotinyl hydrazide on the photoassimilation of glucose.

Gas phase	Compound	Time				
		3 min		15 min		
		Control cpm	INH cpm	Control cpm	INH cpm	INH in 99.97% O_2 cpm
0.03% CO_2 in air	Residual glucose	7254	6724	0	0	2260
	Glycine & serine			1108	2324	3526
	Glycollate	498	912	1252	3128	8415
	Alanine, malate & aspartate	270	64	604	296	200
	Sucrose	5118	3021	10,284	8044	577
	Insolubles	1000	1880	3960	2400	2845
		4 min		11 min		
CO_2 free air	Residual glucose	6998	5634	0	0	
	Glycine & serine			2166	5960	
	Glycollate	828	2300	978	3544	
	Alanine, malate & aspartate	1332	142	2286	254	
	Sucrose	252	362	286	740	
	Insolubles	4082	3540	8175	5675	

These data are consistent with light production of 2-carbon compounds from glucose which may be subsequently metabolised via serine to give either amino acids, e.g., alanine and aspartate, or sugars, e.g., sucrose, according to the experimental conditions.

Evidence for the latter pathway was found when serine-3-^{14}C was fed to Chlorella in the presence of 0.03% CO_2 and 99.97%, the principal end products being sucrose and glycollate. In an attempt to trap a possible intermediate, e.g., PGA, iodo-acetamide was added at 5×10^{-4} M, but this had the surprising effect of increasing the rate of conversion of serine to all compounds.

d) Use of specifically labelled substrates

Further experiments of the type just described were made using radioactive glucose specifically labelled in only one carbon atom. The radioactive

glycollate produced was degraded and the percentage activity in each of the two carbon atoms determined following the method given by Aronoff[12].

Table V. % Distribution of ^{14}C between the carbon atoms of glycollate obtained from specifically labelled glucose-^{14}C.

Gas phase	Additions	% Distribution in glycollate	Time (minutes) 3	8	15	30
CO_2 free 20% O_2	Glucose C_1	C_1	-	10.8	15.8	-
	+ INH	C_2	-	89.2	84.2	-
	Glucose C_6	C_1	-	-	-	21.1
	+ INH	C_2	-	-	-	78.9
	Glucose C_2	C_1	-	-	91.6	-
	+ INH	C_2	-	-	8.4	-
0.03% CO_2 20% O_2	Glucose C_2	C_1	-	-	91.0	92.5
	+ INH	C_2	-	-	9.0	7.5
0.03% CO_2 99.97% O_2	Glucose C_1	C_1	13.8	18.7	24.2	-
		C_2	86.2	81.3	75.8	-
	Glucose C_6	C_1	10.0	19.1	23.8	-
		C_2	84.0	80.9	76.2	-
	Glucose C_2	C_1	-	-	81.8	77.7
		C_2	-	-	18.2	22.3

The results show that C_1 of glucose gives rise to C_2 of glycollate and C_2 of glucose to C_1 of glycollate. Also if C_6 labelled glucose is supplied, the results are essentially the same as when C_1 is fed. Varying oxygen or carbon dioxide partial pressure did not affect these results despite the large changes induced in the production of glycollate. Production of C_2 labelled glycollate from the C_1 of glucose would be consistent with the occurrence of a transketolase reaction involving, e.g., fructose-6-P, and the subsequent liberation of glycollate from a 2-carbon thiamine-pyrophosphate intermediate as has been shown in vitro[12]. However, it is difficult to equate the roles of light and CO_2 with such a mechanism in vivo.

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CHLOROPLAST NUCLEOTIDE COENZYMES

William L. Ogren and David W. Krogmann

While the role of pyridine nucleotides and adenosine triphosphate in photosynthesis is accepted without reservation, there is still a deficit of information concerning the distribution of these coenzymes in the plant. Generally, isolated chloroplasts are incapable of energy conserving reactions unless exogenous coenzymes are supplied. This is reasonably explained by assuming the loss of water soluble material from the chloroplast in the isotonic salt or sucrose solutions used for isolation of this subcellular fraction. Chloroplasts prepared in non aqueous solvents present a possibility for the study of water soluble coenzymes which are associated with the photosynthetic apparatus in vivo. Studies of the distribution of the early products of $C^{14}O_2$ fixation indicate that very little water soluble material is lost from the chloroplasts prepared in organic solvents[1, 2]. Conversely, studies of enzyme distribution patterns suggest that chloroplasts isolated in non aqueous media suffer little contamination with cytoplasmic elements[3, 4]. Nevertheless, so new and unconventional a preparation must be viewed with scepticism. A serious objection to chloroplasts prepared in non aqueous media is the virtual absence of Hill reaction or photophosphorylation activities. The hexane-carbon tetrachloride medium used in cell fractionation extracts much lipid from the chloroplasts - almost all the plastoquinone A, a small amount of chlorophyll and much else. Recently we have succeeded in partially reactivating the Hill reaction activity to indophenol dye by readdition of the crude lipid obtained from concentrating the isolation medium after the chloroplasts have been removed. Representative data are given in Table I.

Table I

Hill Activity of Spinach Chloroplasts
Prepared in Non Aqueous Media

Control	22%
plus PQA	49%
plus PQA, plus extracted lipid	67%

Values are expressed as percentages of the rate of indophenol dye reduction observed with chloroplasts prepared in aqueous 0.4 M sucrose.

Thus non aqueous chloroplasts, when supplemented with appropriate lipids, will carry out the Hill reaction at rates that are comparable to those observed with chloroplasts prepared in the conventional aqueous media.

While data are available on the pyridine nucleotide content of whole leaves and of chloroplasts isolated in aqueous media, a more realistic appraisal of water soluble coenzymes in chloroplasts might be obtained by measurements with chloroplasts isolated in non aqueous medium. In 1954, Anderson and Vennesland had reported values of the TPN and DPN content of spinach leaves[5]. Recently Das and Crane published a value for the TPN content of chloroplasts isolated in isotonic sucrose medium[6]. Using the method of Das and Crane which involves chromatographic separation of the coenzymes and direct enzymatic analysis[7], we have confirmed both these reports and the data are presented in Table II.

Table II

Spinach Pyridine Nucleotide Distribution

	mμmoles per μmole chlorophyll	
	DPN	TPN
Whole leaves	17.4	5.6
Chloroplasts prepared in aqueous 0.4 M sucrose	0.033	0.089
Chloroplasts prepared in non aqueous media	8.87	5.06

It is apparent that a very small fraction of pyridine nucleotide in the leaf appears in the chloroplasts. Data are also presented in this table for the TPN and DPN content of chloroplasts prepared in non aqueous media. This preparation contains approximately half of the total leaf pyridine nucleotides. While most of the leaf TPN is found in the chloroplast, it is not the predominant form of pyridine nucleotide coenzyme in this fraction. This is disquieting in view of the assumptions that TPN is the biosynthetic coenzyme and chloroplasts are biosynthetic organelles.

To exploit the non aqueous chloroplast preparation further, measurements were made of the inorganic phosphate, ADP and ATP levels in the whole leaf and in chloroplasts prepared in non aqueous solvents. The data, obtained by the usual colorimetric measurement of phosphate and enzymatic measurements of ADP and ATP, are given in Table III.

Table III

Inorganic Phosphate, ADP and ATP
Distribution in Spinach

	Whole Leaves	Non Aqueous Chloroplasts
	mμmoles per μmole chlorophyll	
Inorganic phosphate	4.2×10^3	1.15×10^3
ADP	71.1	16.5
ATP	43.3	20.2

In market spinach, about 20% of the total leaf inorganic phosphate appears in the non aqueous chloroplast fraction. Approximately 23% of the total ADP and 46% of the total ATP are found in these chloroplasts which is reassuring in comparison to the negligible quantities of these nucleotides found in chloroplasts prepared in aqueous media.

In the hope that leaves freshly picked and immediately frozen in liquid nitrogen might provide a better representation of the physiological state, red kidney bean plants were used and leaf samples taken from plants held in darkness or exposed to illumination for three hours. The data from a representative experiment are presented in Table IV.

Table IV

Effect of Light on Chloroplast
Nucleotide Coenzyme Levels

	Dark	Three Hours Illumination
DPN	7.21	3.38
TPN	3.91	5.58
ADP	23.9	29.5
ATP	12.3	14.9

mμmoles per μmole chlorophyll

In general, the nucleotide levels of bean leaves are not very different from those found in spinach. However, preillumination causes a distinct shift in chloroplast pyridine nucleotides in that there is a decrease in the DPN and an increase in TPN. A similar shift of DPN to TPN as a result of illumination was observed in whole Chlorella cells by Oh-Hama and Miyachi[8]. Here the shift in form of the coenzyme is seen to occur in the chloroplast fraction and provides some encouragement for the notion that TPN is the preferred coenzyme in biosynthetic or photosynthetic functions. The 20% decrease in total pyridine nucleotide probably reflects

the light induced reduction of a part of the coenzyme pool. The reduced forms of the pyridine nucleotides are destroyed by the acid extraction procedure used in these measurements. While efforts to measure the reduced pyridine nucleotide content are as yet incomplete, the data indicate that there is no more than one fourth as much reduced as oxidized coenzyme in the chloroplast fraction. From this, it appears that the steady state levels of reduced pyridine nucleotide and ATP do not differ greatly in light or darkness.

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METABOLISM OF INORGANIC POLYPHOSPHATES IN GROWING CHLORELLA CELLS

Shigetoh Miyachi

In our laboratory we have been studying on the overall changes in phosphorus compounds in plant cells occuring during photosynthesis. It was found that Chlorella ellipsoidea cells contain large quantities of inorganic polyphosphates. These have been separated into four fractions: poly-P"A" (acid-soluble), poly-P"B" (acid-insoluble but soluble in cold alkali at pH 8-10), poly-P"C" (insoluble either in cold acid or in cold alkali at pH 8-10, but extractable with 2 N KOH at 37° and co-precipitable with potassium perchlorate which is formed upon neutralization of the KOH-extract with perchloric acid (PCA)) and poly-P"D" (extractable with 2 N KOH together with poly-P"C", but different from the latter, does not co-precipitate with potassium perchlorate which is formed upon neutralization of the extract with PCA). In order to understand the role of inorganic polyphosphates in phosphate transfer among the cell constituents, observations were made on the changes of P-distribution occurring in Chlorella grown under photosynthetic conditions or under continued darkness in the presence or absence of an exogenous P-source.

METHODS

The analysis of phosphorus compounds within the algal cells were determined according to published methods[1] with modifications (cf. 2, 3) as follow. The cell material was treated successively with: (I) cold 8% TCA, (II) cold ethanol and hot ethanol-ether, and (III) cold KOH solution of pH 9. The residue thus obtained was (IV) incubated with 2 N KOH at 37° for 16-20 hours, and (V) the supernatant was neutralized with PCA; then (VI) to the supernatant was added PCA to a final concentration of 10%, and (VII) the precipitate formed was suspended in 5% PCA and heated for 20 minutes at 100°.

Orthophosphate was determined by the method of Berenblum and Chain[4] modified by Yanagita[5] using the supernatant obtained by Procedure I.

Acid soluble nucleotidic P compounds were adsorbed by charcoal from the supernatant obtained by Procedure 1. The charcoal was separated by centrifugation and treated with H_2SO_4 (final conc., 1 N) for 20 minutes at 100°. The amount of orthophosphate produced ($\triangle$20 P) was assumed to be nucleotidic labile P.

To the supernatant obtained by Procedure 1 was added a small quantity of carrier polyphosphate, and after adjusting the pH to 4.0, poly-P"A" was precipitated by the addition of Ba^{++}. The amount of poly-P"A" was determined by assaying for orthophosphate liberated from the Ba-precipitate after hydrolysis with 1 N H_2SO_4 for 15 minutes at 100° ($\triangle$15 P). Preliminary experiments(6) showed that the acid soluble polyphosphate in Chlorella cells was completely precipitated by this procedure. It has been also shown that pyrophosphate is not precipitated by this procedure. Poly-P"B" and poly-P"C" were determined by assaying $\triangle$20 P in the supernatant obtained by Procedure III and in the precipitate obtained by Procedure V, respectively. Poly-P"D" was separated from RNA-nucleotides in the supernatant obtained by Procedure VI according to the charcoal treatment: Charcoal was added to the supernatant, shaken vigorously, and the RNA-nucleotides adsorbed on charcoal were removed by centrifugation. Determination of poly-P"D" was done by assaying $\triangle$15 P in the nucleotide-free supernatant. Each polyphosphate, except polyphosphate "C", was purified and identified as long chained polyphosphate (cf. 6,7).

The charcoal separated as above was also treated with 1 N H_2SO_4 for 20 minutes at 100°. P^{32} in the supernatant was a measure of acid-insoluble nucleotidic labile P. Residual P^{32} on the charcoal gave RNA-nucleotide P^{32} (RNA-P^{32}). The RNA-P was also estimated from the absorbancy at 260 mμ in the RNA-fraction.

Lipid-P was assayed by determining the amount of P in the supernatant obtained in Procedure II. The supernatants obtained in Procedure VII were regarded as the DNA fraction. The residue obtained in Procedure VII was regarded as the protein fraction.

RESULTS

Distribution of phosphorus in various compounds

A typical result of analyses is reproduced in Fig. 1. The highest P-content was shown in RNA-P and lipid-P. It should be mentioned, however, that the procedures for the fractionation of the P-compounds

shown were devised to separate the inorganic polyphosphates. It has been found that during the neutralization of the 2 N KOH-extract with PCA (Procedure V, see Methods) not only Poly-P"C" but also an appreciable amount of DNA and protein is co-precipitated with potassium perchlorate, so that the values obtained in these compounds are smaller than they should be. This co-precipitation of DNA and protein can be avoided if the extraction is done with 0.5 N instead of 2 N KOH. On the other hand, poly-P"C" is not precipitated completely during the neutralization of a 0.5 N KOH extract with PCA[8].

Changes of distribution of endogenous P^{32} occurring on incubating P^{32}-labeled Chlorella in P-free medium

Experiment in light - Changes in P^{32}-content in various fractions in normal labeled cells were followed in P-free medium. It has already been reported[7] that the P^{32}-contents in the fractions of DNA and protein increased significantly, while only those in poly-P"A", poly-P"B" and poly-P"C" decreased markedly. As may be seen in Fig. 2, RNA-P^{32} increased while poly-P^{32} "D" decreased steeply. Acid insoluble nucleotidic labile P^{32} decreased slowly. P^{32} in "total RNA-fraction" kept constant or decreased only slightly as reported previously. It was found also that the increase in RNA-P^{32} was accompanied by an increase in UV-absorbancy (E_{260}) as well as of the amount of ribose, confirming that RNA continues to be synthesized under P-deficiency in light. Thus it may be inferred from the present as well as previously published results that, under photosynthetic condition in P-free medium, P used for the syntheses of DNA, RNA and P-protein is supplied from inorganic phosphates.

Experiment in darkness - When some Chlorella cells were incubated in a P-deficient medium in the dark an appreciable decrease of P-content was observed only in the fraction of polyphosphate "B". In parallel with this decrease, an increase of P occurred only in the RNA-fraction, indicating that, under non-photosynthetic conditions, RNA is synthesized with the expenditure of phosphorus of polyphosphate "B". It may be assumed that the mobilization of phosphorus in polyphosphates for the syntheses of DNA and phosphorprotein is a light-induced process, and that in the dark, poly-P "B" serves as the P-donor for the synthesis of RNA, while this process is blocked under photosynthetic conditions[3,9]. In some other cultures of the same species of Chlorella, however, the dark synthesis of RNA with the expenditure of poly-P "B" was not

observed[7]. In most cases poly-P"A" showed a tendency to increase in darkness.

Changes in contents of total P and P^{32} occurring on incubating P^{32}-labeled Chlorella in normal "cold" medium

Experiment in light - The uniformly P^{32}-labeled cells were transferred to the "cold" standard medium, and the changes of distribution of P^{32} as well as of total P in various fractions were followed. In the previous experiment[2] it was shown that the course of increase of total P in the DNA-fraction and in the protein-fraction almost exactly coincided with that of P^{32}, indicating that phosphorus for the synthesis of DNA and P-protein is supplied from the intracellular P-source. As in the P-free experiment, RNA-P, poly-P"D" and acid insoluble nucleotidic labile P were not separated in this experiment but only the total P of these compounds ("total RNA-fraction") were assayed. Data for the above three compounds are reproduced in Fig. 3, and are in accord with the previous observation that phosphorus in the "total RNA-fraction" is supplied almost exclusively from the surrounding solution. It was further shown in this figure that RNA-P^{32} increased only slowly whereas RNA-total P, as calculated from the UV-absorption, increased steeply, and confirmed our previous conclusion that during photosynthesis the phosphorus used for most of the RNA taken from the extracellular P source, although some RNA is supplied through intracellular phosphorus. P^{32} in poly-P"D" and nucleotidic labile P did not decrease or decreased only slightly although they took up phosphorus from the surrounding medium. In Fig. 4 are shown results demonstrating the changes in total P and P^{32} in other polyphosphates. (For technical reasons (because of the addition of carrier polyphosphate in the assaying procedure) the total P content in poly-P"A" cound not be determined.) As may be seen in the figure, P^{32} in poly-P"A" and poly-P"C" always decreased steeply, whereas P^{32} in poly-P"B" decreased only slowly or did not decrease in some cases. From these results it may be assumed that only poly-P"A" and poly-P"C" are functioning in the mobilization of P to other intracellular P-compounds such as DNA and P-protein under normal photosynthetic conditions. Poly-P "B" and poly-P"D" are functioning as P-reservoirs which are used only under conditions of P-deficiency.

It was also observed that the decrease in poly-P^{32} "A" is usually preceded by a transient increase. It may be inferred from this finding that poly-P"A" accepts and transfers P from and to other intracellular P-sources. Although it was not shown in the figure, P^{32} in the lipid

fraction showed only a slight increase, confirming our previous conclusion that most of P for the synthesis of P-lipid is supplied from the external medium.

Experiments in darkness - Using the cell material which cannot synthesize RNA in the dark in P-free medium, it was found that no change was observed in the amount of total P or P^{32} in the respective cellular P-compounds except the gradual increase in poly-P^{32} "A". From these results as well as those described above it may be concluded that mobilization of P from an intracellular P compound to poly-P"A" is a light-independent process while the transfer of P from polyphosphates to other cellular P-compounds such as DNA and P-protein is a light dependent process.

Incorporation of phosphate under normal photosynthetic conditions or in the dark

Chlorella cells which had been growing in the "cold" normal medium were supplied with P^{32}-labeled phosphate under photosynthetic conditions or in the dark, and the subsequent process of incorporation of P^{32} by the cells into poly-P"B", poly-P"C", labile nucleotides, lipid, DNA and protein was followed for comparatively short periods (0.5-8 hours). It was found that the synthesis of poly-P"C" occurs only in the light whereas that of poly-P"B" and P-lipid takes place independent of light at least for a limited period.

DISCUSSION

This study has revealed that under normal photosynthetic conditions, P for the syntheses of intracellular P-compounds such as DNA and P-protein is supplied from poly-P"C" and poly-P"A" whereas that for the syntheses of RNA and P-lipid is supplied from extracellular orthophosphate without the intervention of polyphosphates. Most of poly-P"B" and poly-P "D" are not normally mobilized. Under P-deficiency in the light, however, synthesis of not only DNA and P-protein but also RNA is maintained with concomitant decrease of all kinds of polyphosphates. These observations indicate that poly-P"B" and poly-P"D" are P-reservoirs which are used only under P-deficiency. It is assumed these polyphosphates are degraded to orthophosphate before transfer to other cellular P-compounds.

One might assume that the acid-insoluble nucleotidic labile P is a hydrolysis product(s) of stable RNA-nucleotides. But the facts that

stable P^{32} in RNA-nucleotides (RNA-P^{32}) increases slowly while nucleotidic labile P^{32} decreases slowly under photosynthetic condition indicate that the nucleotidic labile P is not a simple hydrolysis product of RNA but it may be labile P-compound(s) combined with nucleotides. We have, however, no evidence to judge whether this labile compound is inorganic polyphosphate or not.

Our study revealed that Chlorella cells contain at least two groups of polyphosphate which function differently; one group, poly-P"C" and poly-P"A", plays a role as intermediate for the syntheses of intracellular P-compounds which transfer P to the substances such as DNA and P-protein under normal condition, and another group, poly-P"B" and poly-P"D", functions as P reservoir but not as an energy reservoir[10,11], as concluded by Harold[12] for Neurospora.

Little is known of the path by which P from poly-P"C" and poly-P"A" is transferred to other cellular compounds such as DNA and P-protein, except the fact that the mobilization takes place only in the light. ADP is generally considered to be the acceptor of P from polyphosphates. It may also be possible, however, to assume the direct transfer of P from polyphosphate. The recent discovery by Szymona[13] with Mycobacterium of an enzyme which catalyzed the formation of glucose-6-P from polyphosphate and glucose suggests at such a possibility.

One may assume that photosynthetic or respiratory ATP is the precursor of polyphosphate in Chlorella. In this connection, Harold found that the accumulation of polyphosphate caused by the addition of phosphate to phosphate-starved mycelium of Neurospora is always preceded by ATP accumulation and that the turnover of this compound is very rapid, supporting the view that ATP is the metabolic precursor of polyphosphate in this organisms. The data we obtained indicate that, at least a part of poly-P"A" is not synthesized directly from extracellular orthophosphate but through intracellular P-compounds and the process is light-independent. By our previous study with synchronized Chlorella cells[14], it was found that the amount of P incorporated into poly-P"A" and some other P-compounds from other intracellular P-compounds increases gradually with the progress of culture, attains the maximum level at the stage of ripening, and decreased markedly during the process of "post ripening" and "division" of cells indicating that these compounds are playing an important role in the process of cell maturation and division. As the concomitant decrease of P with the increase of poly-P"A" was observed only in poly-P"C", it was inferred that poly-P"A" received P from poly-P"C". In our dark experiment, however, decrease of poly-P^{32}"C" concomitant

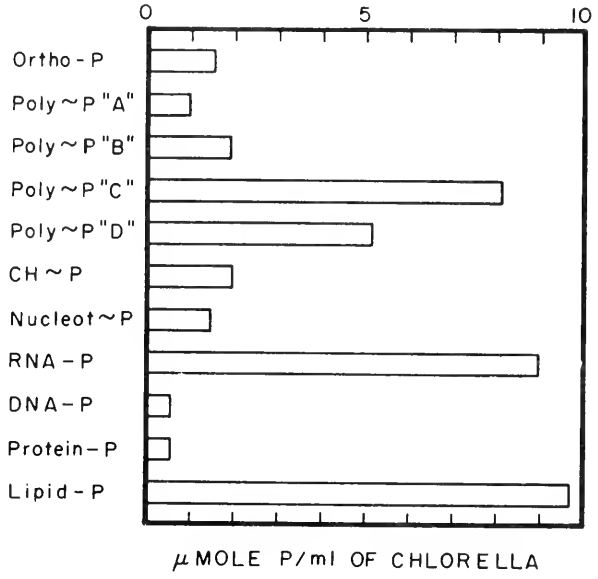

Fig. 1. Distribution of P in various fractions

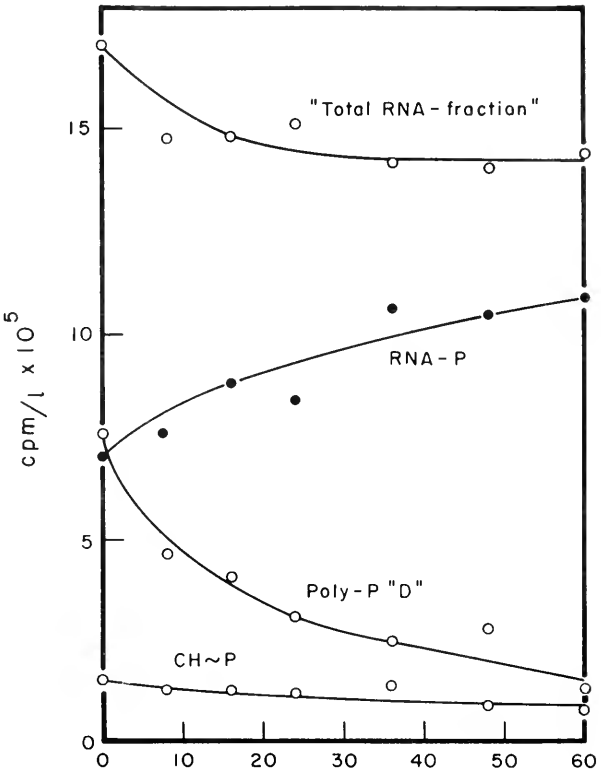

Fig. 2. Hours in P-free medium in light

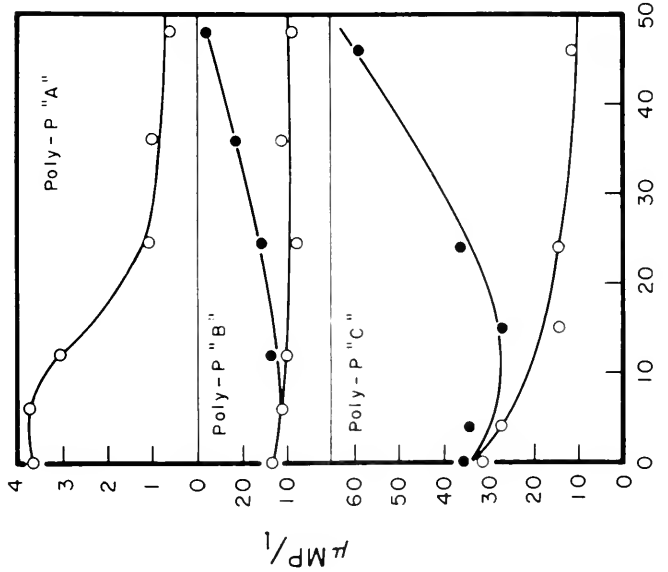

Fig. 3. Hours in "cold" medium in light

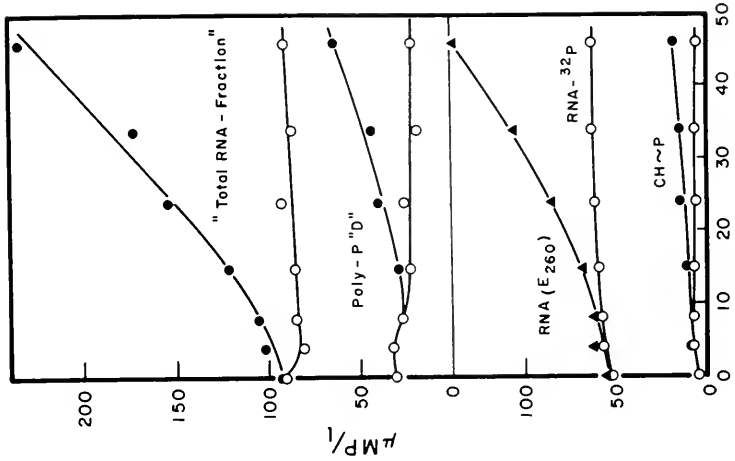

Fig. 4. Hours in "cold" medium in light

with the increase with poly-P^{32} "A" could not be detected, because the increment in P^{32} in poly-P^{32} "A" itself is too small as compared with the amount of poly-P"C". By the short term experiment it has been found that incorporation of orthophosphate-P^{32} into poly-P"B" and lipid-P takes place independent of light at least for a limited period, whereas incorporation of ortho-P^{32} into poly-P"C" occurs only in light[3]. The schematic representation on the formation and transformation of respective polyphosphates and their probable relationship to the syntheses of nucleic acids, P-protein and P-lipid as elucidated thus far is shown in Fig. 5.

The responses of polyphosphates to light seems to indicate different locations of each polyphosphate within the Chlorella cell. From the facts that the formation of poly-P"C" and the mobilization of P in poly-P"C" for the syntheses of other P-compounds are induced by light, we are tempted to presume that it exists in or very near to the chloroplast. Our preliminary study with Chlorella[15] showed that the volutin granule consists of poly-P"A" and RNA but it could not be decided whether poly-P"C" exists in chloroplasts or not, although labile P-compounds were detected in the poly-P"C"-fraction of chloroplasts. It has been also found that acid-insoluble inorganic polyphosphate exists in spinach leaves[16]. This suggests that the presence and function of polyphosphates are not limited to lower plants but they are playing an active role in the photo-induced intracellular phosphate transfer in green cells in general.

ACKNOWLEDGMENT

The research reviewed in this paper has been the result of collobora-tion with Prof. E. Hase, Dr. R. Kanai, Miss S. Mihara, Mr. S. Aoki and Mrs. S. Miyachi.

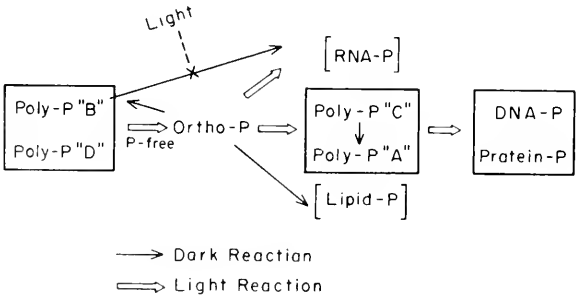

Fig. 5. Incorporation and mobilization of P in Chlorella cells

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X. MISCELLANEOUS TOPICS

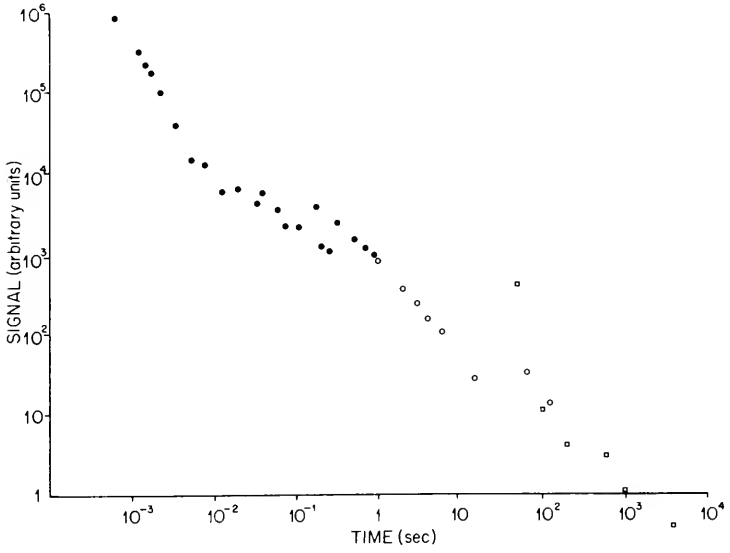
THE DECAY OF DELAYED LIGHT AT SHORT TIMES

William Arnold and J. B. Davidson

In this brief report we would like to present some preliminary results of measurements of the decay of the delayed light emission from Chlorella in the time region of 10^{-5} to 10^{-3} seconds. Although the experiments are still in progress, we feel that the data are of sufficient interest to warrant presenting them to this Symposium.

The results were mainly obtained with two devices which will be described in detail in a future paper. One is a phosphoroscope patterned after one used by Dr. R. S. Becker at the University of Houston. It covers the range of from 0.65 to 1000 milliseconds. The second device is a shutter consisting of a small piece of 0.25 mil aluminized Mylar film operated by the blast from a carbon dioxide pistol. This shutter was used in the 50 to 1000 microseconds range.

Figure 1 is a composite decay curve for Chlorella at 25° C.



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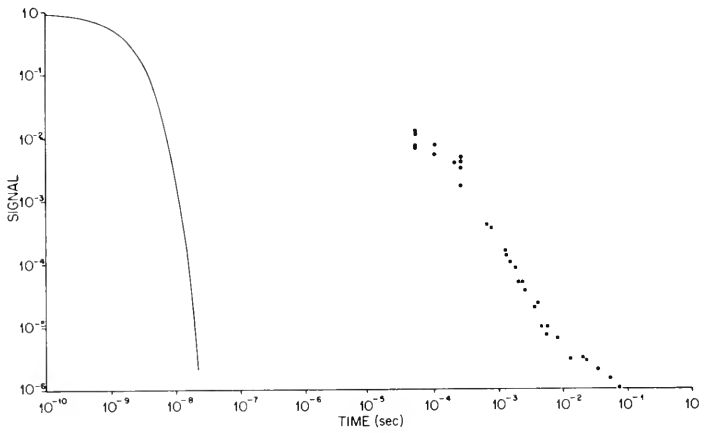
The data, over the region of 0.65 milliseconds to 0.9 seconds, which were taken with the phosphoroscope has been combined with two other sets of points, determined on older, slower equipment. The ordinates of the different decay curves have been multiplied by constants so that they match at the points of overlap. White light was used for illuminating the cells. All points, with the possible exception of those at the shortest times, were taken at "light saturation." It should be emphasized that throughout the entire decay of the delayed light, the intensity of the exciting light needed to give a maximum signal is increased as the time in the dark is decreased.

Figure 1 also shows that over the range of 6.5×10^{-4} to 4×10^{3} seconds the delayed light signal is approximately proportional to 1/t.

Figure 2 is also a decay curve for delayed light from Chlorella at 25° C. Here the exciting light was blue as obtained from a tungsten lamp through a filter of 10 cm of saturated copper sulfate. The lamp was operated so that the delayed light signal was proportional to exciting light intensity. A red filter eliminated the exciting light from the photomultiplier.



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The phosphoroscope and the fast shutter were arranged so that fluorescence as well as delayed light could be measured. The delayed light signals are plotted in the figure as fractions of the steady state fluorescence signal, which is taken to be unity. The points at 0.6 milliseconds and longer times were taken with the phosphoroscope. The points for the shorter times were made with the Mylar film shutter. The solid curve plotted at the left for reference is an exponential having a time constant of 1.7×10^{-9} seconds, which is the lifetime for the fluorescence of Chlorella as given by Tomita and Rabinowitch.

From Figure 2 it can be seen that intensity of the delayed light at 5×10^{-5} seconds is approximately one percent of the fluorescent intensity.

Extrapolation of the decay curve to even shorter times suggests that a large fraction of the in vivo fluorescence of green plants may actually be delayed light.

EFFECTS OF PHOTOSYNTHETIC POISONS ON DELAYED LIGHT IN THE MILLISECOND TIME RANGE

Walter F. Bertsch, J. B. Davidson, and J. R. Azzi

The initial steps in photosynthesis involve conversion of energy from light quanta into some form that is available for utilization in the biochemical reduction of carbon dioxide. Since about eight light quanta are needed to reduce one carbon dioxide molecule[16] and since the absorption cross section for photosynthesis is known to be the size of several hundred chlorophyll molecules[22], it is clear that some type of cooperation exists between chlorophyll molecules in the photosynthetic apparatus. A great deal of evidence indicates that this cooperation occurs within groups of chlorophylls, known as photosynthetic units[14, 15, 18, 19, 22-24]. The energy from a photon absorbed within a photosynthetic unit presumably migrates by resonance transfer[5, 9, 13, 17] until it reaches a reaction center. At the reaction center the energy is somehow made available to run biochemical dark reactions.

It is now known that these early steps in quantum conversion involve purely electronic, as well as enzymatic, processes[2, 12]. Furthermore, the photosynthetic apparatus of higher plants and photosynthetic bacteria, when dried, has electrical properties that are usually associated with organic semiconductors—photoconduction, thermoluminescence, and increased conductivity at higher temperatures[4, 6, 7]. It therefore seems that the delayed light emission of living plants[28] might provide a direct physical measurement of electron transitions involved in photosynthetic quantum conversion.

The dim glow from plants is emitted by the first excited singlet transition of chlorophyll[3, 8, 31, 32] and has decay characteristics that may be interpreted in terms of untrapping of electrons in a semiconductor[1, 11, 31]. However, it is not certain that the delayed light is actually produced by a solid state physical phenomenon of this sort.

Regardless of the physical mechanism of delayed light emission, if the delayed light is associated with photosynthesis, it is presumably emitted from very early steps of quantum conversion. The argument is simple: the

emitted quantum contains essentially all the energy of the original absorbed quantum, so that there would be no possibility of emission if the energy had been degraded by one or more reactions.

It therefore appears that an understanding of the delayed light emission might provide new insight into the mechanism of photosynthetic quantum conversion. However, to assess the significance of the delayed light measurements, we must determine whether the emission is closely associated with photosynthesis.

The delayed light emitted at times longer than about 0.1 sec after illumination is indeed intimately related to photosynthesis. There is ample evidence for the involvement of photosynthetic enzymes with the emission in this time range [26, 27, 29]. In addition, the delayed light at these longer times is emitted from functionally active chlorophyll, since the presence of two photosynthetic pigment systems can be deduced from the direct evidence of chromatic transients, and of changes in the time course of emission caused by excitation at different wavelengths[10, 20, 21].

In the millisecond time range, much less evidence has been given for direct involvement of the photosynthetic apparatus in delayed light emission. Sweetser et al.[29] reported some effects of poisons on delayed light emission from 4 msec to longer times. The present paper gives additional information about the effects of photosynthetic poisons on the fast delayed light decay. We have measured the emission from 1 to 20 msec after illumination. Of those poisons used, every one that was known to act on the first steps of photosynthesis had a significant effect on the delayed light emission. The poisons may be classed into several groups according to the way they affect delayed light. These data imply that delayed light in the millisecond range is emitted from functionally active chlorophyll.

MATERIALS AND METHODS

The delayed light emitted from 1 to 20 msec after the middle of an illuminating flash was measured with a phosphoroscope-photomultiplier-oscilloscope apparatus similar to one used by Dr. R. S. Becker at the University of Houston. This instrument, built by one of us (J. B. D.) in collaboration with Dr. W. A. Arnold, will be described in a later publication. The exciting light was a 1000-watt tungsten bulb, filtered by 8 cm. of 90% saturated $CuSO_4$ solution, with the filaments focussed on the cells to give an image of about 4^1 cm^2.

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Exponential phase cultures of Chlorella pyrenoidosa strain 252, obtained from the Culture Collection of Algae, Indiana University, were used in all experiments. These cultures had been grown at 18° C in Knop solution aerated with 5% CO_2–95% air under continuous illumination from neon arcs.

RESULTS

Figure 1 shows the 1.5 to 8 msec decay of delayed light emission from unpoisoned cells.

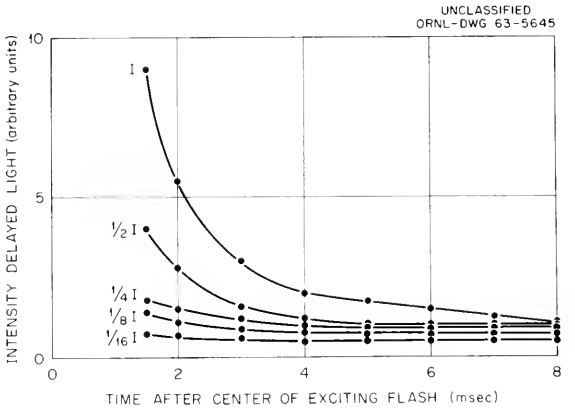

Figure 1. Effect of excitation intensity on delayed light emission from 1.5 to 8 msec. Fast components in the decay are present only after high intensity excitation.

The time course of decay depends on the intensity of exciting light. Since the fast components of delayed light emission are present only at very high intensities of illumination, we have used the highest intensity shown in Figure 1 in our experiments.

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Cyanide is known to inhibit the dark reactions of photosynthetic CO_2 reduction without affecting the quantum conversion steps[15, 25, 33, 34]. We found no effect of KCN (10^{-9} to 10^{-2} M) on the 1 to 20 msec delayed light emission.

Two poisons changed the intensity of delayed light emission without significantly changing the time course of decay; azide and 2-chloro-6-nitrophenol. Figure 2 gives the effect of NaN_3 (10^{-5} to 10^{-1} M) on delayed light emission at various times. The curves for the various times are approximately proportional to each other, implying that the poison had the same relative effect at each time of emission. Azide may therefore be said to inhibit delayed light without changing the shape of the decay curve. 2-chloro-6-nitrophenol also had a relatively small effect on the time course of delayed light emission, but this poison increased the intensity of emission. Figure 3 shows that at optimal concentrations of 2-chloro-6-nitrophenol (7 to 10×10^{-5} M) the emission at 1 msec was increased by a factor of about 3.5, and at 20 msec by a factor of about 7. Sweetser et al.[29] also reported that 2-chloro-6-nitrophenol increased delayed light emission, and also that it was a powerful inhibitor of photosynthesis.

Several poisons that are believed to act very close to photochemical steps were tested. These compounds had a marked effect on the time course of delayed light emission, as well as on the intensity of emission. These poisons may be put in two categories, according to their effect on the time course.

1. Diuron (DCMU), monuron (CMU), orthophenanthroline (all at 10^{-7} to 10^{-3} M) had similar effects on the emission. At certain concentrations they resulted in a very flat decay curve in which no fast decay components were present in the 1 to 20 msec time range. As the concentration of each poison was increased, the decay remained flat but was reduced in intensity. The lowest concentration at which the fast decay components were strongly inhibited without reducing the intensity of emission were different for the individual compounds: 5×10^{-6} M for DCMU, 10^{-5} M for CMU, and 10^{-3} M for orthophenanthroline. The delayed light decay of unpoisoned cells is compared to that of CMU-treated cells in Figure 4 (top and bottom). The effect of various concentrations of orthophenanthroline is shown in Figure 5 for various times of emission. At 10^{-3} M this poison slightly inhibited the 1 msec emission while increasing the 20 msec emission by a factor of 15.

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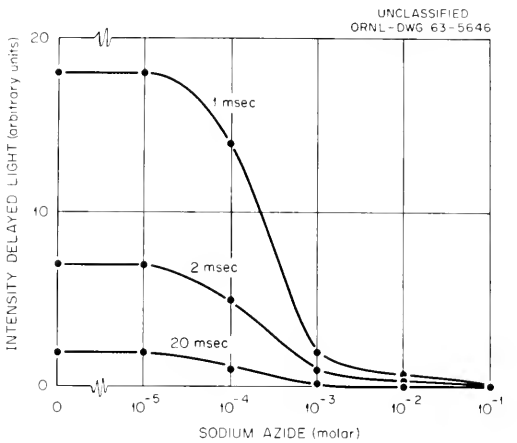

Figure 2. Reduction caused by azide in delayed light emission. Sodium azide reduced the intensity of emission without significantly changing the time course of decay.

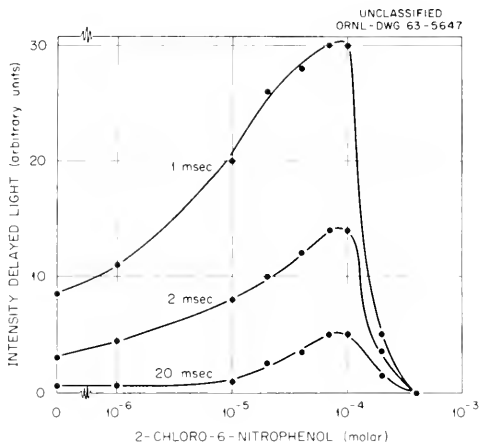

Figure 3. Increase caused by 2-chloro-6-nitrophenol in delayed light emission. 2-chloro-6-nitrophenol (up to 10^{-4} M) increased the intensity of emission without significantly changing the time course of decay.

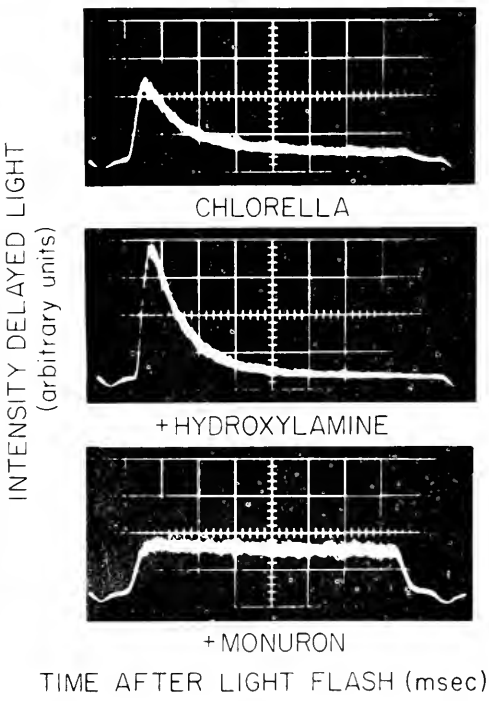

Figure 4. Effect of hydroxylamine and monuron on delayed-light emission from 0.6 to 4 msec. The emission as presented on the oscilloscope screen was photographed for unpoisoned Chlorella cells, cells poisoned with hydroxylamine sulfate (10^{-3} M), and cells poisoned with monuron (10^{-4} M). Vertical grid indicates time after middle of exciting flash in 0.5 msec per unit, horizontal grid indicates intensity of delayed light emission in arbitrary units (5 millivolts per unit on top and middle photographs, 2 millivolts per unit on bottom photograph). Center of exciting flash is indicated by the small pip in the zero line on either side of the decay curve. The decay from unpoisoned cells fits neither monomolecular nor bimolecular kinetics. Poisoning with hydroxylamine resulted in a nearly exponential decay. Poisoning with monuron resulted in a flat decay without fast components.

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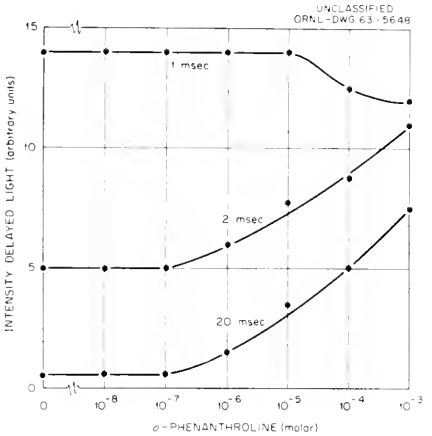

Figure 5. Change caused by O-phenanthioline in delayed light emission. O-phenanthioline caused a loss of fast decay components by strongly increasing the intensity of emission at longer times.

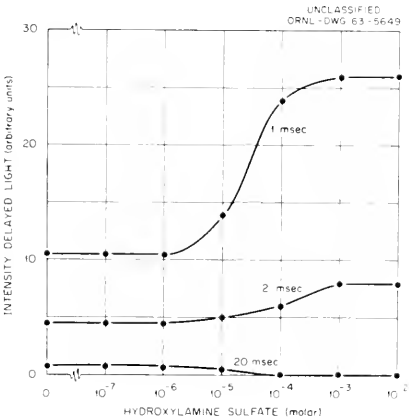

Figure 6. Change caused by hydroxylamine in delayed light emission. Hydroxylamine caused an almost exponential decay by increasing the intensity at short times and decreasing intensity at longer times.

2. Hydroxylamine (10^{-7} to 10^{-3} M) at higher concentrations results in a decay that approaches first order kinetics. From 1 to 8 msec (emission at longer times was too dim to measure) cells poisoned with NH_2OH (10^{-4} M) gave a decay that was very nearly exponential. This decay is shown in Figure 4 (middle) for comparison with unpoisoned cells and with CMU-poisoned cells. Concentration response curves for hydroxylamine sulfate are given in Figure 6, which shows that at 10^{-3} M the emission at 1 msec was increased by more than a factor of 2, while the 20 msec emission was too small to be observed with our present apparatus.

DISCUSSION

Since the shape of the delayed light decay curve depends on intensity of exciting light (Figure 1), statements concerning the kinetics of the decay are valid only in terms of the intensity of excitation. Our data at saturating intensities of exciting light indicate that the decay from unpoisoned plants has neither monomolecular nor bimolecular kinetics, in agreement with Tollin et al. (31).

The differential actions of the poisons which we tested indicate that the delayed light at 1 to 20 msec must be emitted from functional chlorophyll that is involved in photosynthetic quantum conversion. That is, every poison used which was known to act on the first steps of photosynthesis had a strong action on the 1 to 20 msec emission, whereas the poison (cyanide) that is known to have no action on the first steps had no effect whatever on emission in this time range.

All the poisons which affected delayed light presumably act on reactions involved in photosynthetic quantum conversion. We suspect that those compounds that affected the intensity of emission without strongly affecting the time course of delay (azide, 2-chloro-6-nitrophenol) are not acting on steps that are adjacent to photoreactions. These compounds seem to change the number of electrons that may potentially emit delayed light without affecting the processes by which the emission takes place. Poisons which drastically changed the time course of emission (DCMU, CMU, orthophenanthroline, hydroxylamine) must act on steps which are closely associated with the emitting chlorophyll system. The extremely flat decay, and the 5- to 15-fold increase in intensity at 20 msec caused by DCMU, CMU, and orthophenanthroline, suggest that these compounds may block electron or hole flow out of the

chlorophyll system. Hydroxylamine may also act in this manner, but the very different decay curve caused by this poison implies action at a different site from the other three poisons.

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LIGHT SCATTERING BY CHLOROPLASTS IN THE UV

William F. Prickett, F. Dudley Bryant, and Paul Latimer

Scattering spectra of biological materials which contain light absorbing compounds are closely related to the absorption spectra. Several green plant materials and red blood cells have been studied in the visible spectrum. However, the present study[1] seems to be the first of a biological material in which a scattering spectrum is measured in the important near ultraviolet.

The method is described in detail elsewhere[2, 3]. Briefly, scattering spectra are determined from the difference between two "absorbance spectra" of a given sample measured with a standard spectrophotometer (we used the B & L 505). In one case the optics of the instrument are used in the normal configuration; in the other diffusing plates are placed after the sample and blank vessels. From particle (chloroplast) concentration, particle size, and other information, absolute scattering coefficients are calculated. While only light scattered at small angles, 5 - 49°, is observed, these coefficients are extrapolated to describe total scattering (assuming that light scattered in all directions has the same spectral properties). The scattering coefficient, S_p, is the fraction of the incident light striking the geometrical cross section of the particle which is scattered. From the above experimental information and using the equations and method of Latimer and Eubanks, we also calculate the absorbance, E'_p (corrected for scattering losses) of a single particle ($E'_p = \log I_o/I$).

Spinach chloroplasts in 0.5 M sucrose buffered at pH 7.0 were obtained with a blender, washed twice, and resuspended for measurements in standard 1 cm^2 silica vessels. To cancel out errors caused by dissolved proteins and small particles in the sample vessel, supernate of the stock suspension was added to the blank medium in the appropriate concentration. We made UV diffusing plates by grinding Corning No. 7910 glass on both sides with No. 100 carborundum.

The results of two consistent runs were averaged to obtain the given spectral curves. The average particle diameter was 3.0 μ, average particle concentration, N, was 1.35×10^7/ml (P = 0.94, see Ref. 3). Experimental suspension absorbances were in the range 0.1 - 0.4 depending on sample, wavelength, and type of measurement.

The particle scattering and absorbance curves are given below in Fig. 1. Of particular interest is the scattering shoulder at about 280 - 305 mμ corresponding to the broad absorbance band of proteins and nucleoproteins at 255 -

280 mμ. Also seen is a well defined scattering band at about 347 mμ which is apparently related to the DPNH band at 340 mμ. Other structure is seen in the scattering curve although it is not very much greater than random error.

Although the experimental data extended from 240 - 460 mμ, only a portion of it is shown on the figure. At the shorter wavelengths, the scattering curve simply rises sharply. At the longer wavelengths it agrees with a previously reported curve.[2]

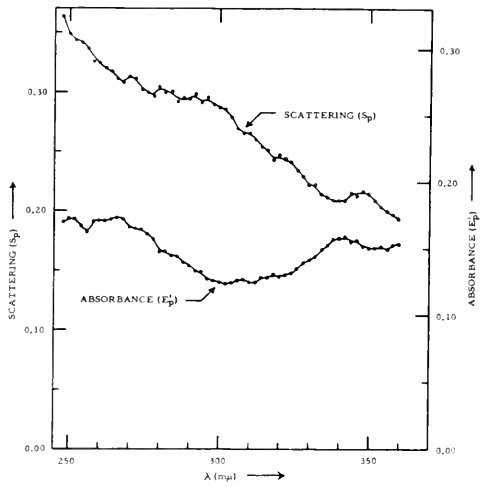

Fig. 1. Spinach chloroplast: absolute absorbance and scattering cross section.

The only rigorous light scattering equations which have been used to account for scattering spectra of biological particles are obtained from the so called "large particle" postulates[4, 5]. The particles are assumed to be large compared with the wavelength of the incident light and without internal scattering structures so that the simple laws of geometrical optics apply within the particle. The difference between the refractive index of the particle and that of the surrounding medium is assumed to be small, thus permitting surface reflection and refractive bending to be neglected. In this case, scattering can be explained in terms of destructive interference in the forward direction, and constructive interference in the other directions, between light passing through the particle and that passing around it.

Several authors have used this type of equation to qualitatively account for experimental scattering and extinction curves of algae[2, 6, 7, 8] and red blood cells[5, 9]. In studies of oriented red cells which are relatively large ($\sim$8 μ diameter) and devoid of internal structure, the quantitative agreement between this type of theory and experiment is reasonably good in the spectral region near $\lambda = 0.4$ μ (diameter/wavelength in water $\cong$ 25). Such equations may also be applicable to the chloroplasts (3 μ diameter) in the UV ($\lambda \cong .3$ μ).

However, we did not use them to attempt to account for the scattering curve in Fig. 1 because the refractive index at these wavelengths is not sufficiently well known. As discussed elsewhere[2], this large particle theory predicts that the relation between the scattering and absorption spectra will depend - amongst other things - on particle size. For some sizes (e.g., small particles) the scattering curve is S - shaped near the absorption maximum as is the dispersion curve[10]. For other particle sizes, the predicted shape of the scattering curve will be that of an inverted absorption curve (minimum scattering at wavelengths where maximum absorbance occurs). The above scattering spectrum seems to be a combination of these forms.

While "large particle" equations successfully predicted certain optical properties of red blood cells, a more severe test is obtained by applying one of them to scattering by chloroplasts (diameter 3 - 4 μ) near the red chlorophyll maximum, $\lambda = 0.68\ \mu$ (diameter/wavelength in water $\cong 7$). Not only are the particles smaller, compared with the wavelength, than are red cells but they have more internal structure. A suitable spectral scattering curve and the needed auxiliary experimental information for spinach chloroplasts have been reported[2, 3] and in the following paragraphs we use this to test the theory. The data is very similar to that which we recently obtained using a special spectrophotometer and partial single layers of chloroplasts.

All needed information about the particles is available except definite information about the structure of the chlorophyll a band in vivo. (Does chlorophyll a exist in vivo in one, two, or more forms with different band maxima in this region?) The absorption band structure is needed for the dispersion theory calculations of the dependence of particle refractive index on wavelength. Since the structure of this band is not known with certainty, we calculated three dispersion curves and used each to calculate a scattering curve for comparison with the experimental scattering curve. Dispersion curves were calculated from band parameters which in turn were obtained by trial fittings of a corrected experimental absorbance curve. In this fitting, a basic model of the band structure is assumed. Then all band parameters (position, height, and width) are adjusted, automatically on a computer, to obtain the best fit. In all resulting fits a chlorophyll b band was assumed at 654 mμ and also minor bands at shorter wavelengths. In case I, the major chlorophyll a in vivo band was assumed to be a single Gaussian shaped curve with a maximum at 676 mμ. In the second case, II, the band was assumed to be composed of two spectroscopically distinct forms present in about equal concentrations with band maxima at 671 and 682 mμ. Finally in case III a third band was added at 697 mμ, its magnitude being about 10% of the sum of the 671 and 682 mμ bands. Our scattering equation leading to S_p - values used here was a modified version of Eq. 10 of Ref.(5). The three resulting theoretical scattering curves and the experimental curve[2] are shown on Fig. 2.

While there is qualitative agreement between theory and experiment, the large particle equation predicts much less dramatic selective scattering than actually occurs. Furthermore, the absolute theoretical scattering cross section is of the order of 1.0 while the experimentally observed cross section is

about 0.3. Hence this approximate type of theoretical approach is quantitatively inadequate for these chloroplasts in the red region in contrast to the finding for red blood cells[5] in the blue spectral region. The present failure is probably because of the low ratio of particle size to wavelength and the neglected effects of internal particle structure.

This finding differs from that of Charney and Brackett[8] who obtained surprisingly good agreement between the predictions of similar equations and experiment. Part of the reason for this disagreement lies in the assumed effects of suspension attenuation on the shape of the scattering spectrum. Their

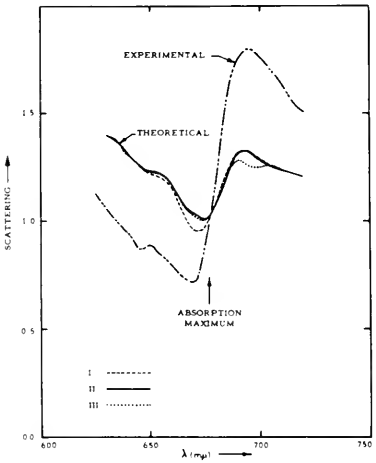

Fig. 2. Spinach chloroplast: scattering cross section (S_p), experimental and theoretical. Ordinate scale is absolute for theoretical curves. Experimental curve was multiplied by 5.4 for plotting convenience. Theoretical curves are based on one, two, and three band models of chlorophyll a system.

correction for attenuation greatly enhances the ratio: maximum to minimum values on the theoretical scattering curve. While it appears to have been appropriate in accounting for their experimental data, no correction for such effects was made on our theoretical curve since the experimental method in principle yields curves which are corrected for the attenuation. In addition, it should be noted that our recent studies of single partial layers of similar chloroplasts gave experimental scattering curves with a somewhat greater ratio of maximum to minimum than is seen on the above experimental curve. Because of the similarity of particle parameters, the theoretical curves for these latter chloroplasts should be similar to that given above. Since it is clear that no attenuation correction is appropriate in this case, this confirms that the experimental scattering curve in Fig. 2 is representative of particle characteristics unmodified by distorting influences. Thus it is appropriate to compare this with theoretical predictions of particle characteristics.

However, despite this limitation of these theoretical curves, which depend on assumed absorption band structure, further examination suggests that measurements of scattered light may be capable of providing information about

absorption band structure. There is indeed precedence for this type of approach to absorbance spectroscopy[11]. While the theoretical scattering curves I and II, based on the assumption of one and two components only of the chlorophyll a band, give very similar theoretical scattering curves, we note that scattering curve III, based on the assumption that there is a significant band at 697 mμ, clearly reflects the presence of this absorption band. No experimental scattering spectrum to date has shown clear evidence of such a band.

The present data is not sufficiently precise to exclude the existence of a small chlorophyll a band in vivo near 700 mμ. However, this neglected approach to absorbance spectrophotometry of living materials warrants further exploration. More precise measurements of scattered light are needed.

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ADDENDUM
(added during the meeting)

At this meeting Dr. R. A. Olsen gave further evidence of the presence in chloroplasts of a pigment with an absorption maximum near 700 mμ. His studies of absorption and fluorescence indicate that molecules of this pigment are highly oriented with respective dipoles being parallel to one another. We here give other evidence of this pigment based on a new interpretation of previously reported double refraction spectra of Mougeotia cells.

Goedheer and also Menke and Menke (see Ref. 21 and 22 of the above Ref. 6) reported spectral birefringence curves which display strong anomalous dispersion effects caused by the chlorophylls. Such effects can be produced by two mechanisms: one, by the pigment molecules residing only in every other layer of the granum (form birefringence), and two, by the pigment molecules being spacially oriented (orientation birefringence). On a per molecule basis, the orientation mechanism is much more efficient. It has been frequently assumed that these authors' curves were produced by form birefringence only. However, especially in the case of mature cells, the spectral birefringence curves are symmetrical about a wavelength near 690 mμ instead of near 675 mμ, the nominal position of the main chlorophyll maximum. There are no conditions under which dispersion theory can predict a shift of the center of an anomalous dispersion curve away from the absorption maximum of the pigments producing it. Hence the birefringence curves must have been strongly influenced by pigment molecules absorbing near 700 mμ.

The most likely explanation is that the observed birefringence is caused by a combination of the above sources. The form birefringence is produced by the predominant 675 mμ band (or components thereof). Molecules of this band are not spacially oriented as indicated by Olsen. The orientation birefringence is produced by the less prevalent but highly oriented molecules with an absorption maximum near 700 mμ. Our approximate calculations, based on the above data, suggest that the concentration of this latter pigment can be as high as 10% of the total pigments producing the 675 mμ band (this assumes a 700 mμ molecule with the extinction coefficient of chlorophyll a). Perhaps this 700 mμ pigment is that of case III above, the presence of which in spinach chloroplasts was indicated by our approximate absorption spectrum analysis.

INTERFERENCE OF EMISSION CHANGES WITH FAST ABSORPTION CHANGES IN THE FLASH SPECTROSCOPY OF ALGAE

Edgar Inselberg and J. L. Rosenberg

Many significant findings have emerged in recent years from the flash spectroscopy of algae, notably from Witt's and Kok's laboratories.

Our own group constructed a flash apparatus at a later stage and studied some aspects of the 515 mμ change found by Witt and Moraw (6). As interest shifted to changes in the 700 mμ region, first reported by Kok (2), we modified our apparatus for measurements in the far-red region.

EXPERIMENTAL

The apparatus is illustrated in Fig. 1. The monochromatic beam (10^2 - 10^3 erg $cm^{-2}sec^{-1}$ in intensity, depending on the experiment) is focused into the sample, and then the transmitted fraction of the beam is detected by a red-sensitive multiplier phototube (RCA 7102). At right angle to the monochromatic beam, the sample is excited by short flashes from a General Electric FT-230 flashtube (18.5 joules input per flash). Filter F_1 determines the spectral region of excitation, while F_2 excludes all but a small fraction of the actinic light from the phototube. The output of the phototube, corresponding to single flashes, is displayed on an oscilloscope screen.

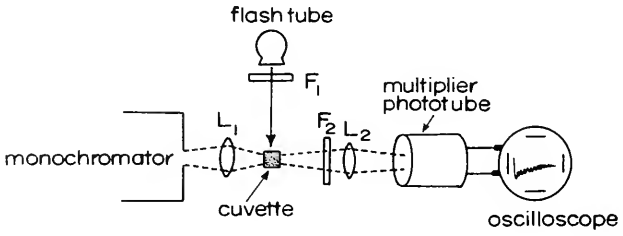

Fig. 1. Top view of the flash apparatus.

Edgar Inselberg and J. L. Rosenberg

Several investigators have considered the possibility that luminescence may interfere with measurements of fast absorption changes in the far-red region: for example, Witt's apparatus (5) provided for an additional phototube, at right angle to the monochromatic and actinic beams, to measure fluorescence; Kok (3) allowed for a delay of about one millisecond after the flash was extinguished before making measurements. We had originally assumed that luminescence resulting from the actinic light would, essentially, be additive to the luminescence from the monochromatic beam. Accordingly, we were using as a blank the waveform of the flash (fraction transmitted by F_2), which would include luminescence caused by the actinic light. Moreover, the oscilloscope trace was zeroed about 20 seconds after the monochromatic light was turned on, prior to flashing the sample; by then the trace had been adequately stabilized, indicating that the fluorescence intensity due to the monochromatic light could be considered unchanged over the brief excitation period.

RESULTS AND DISCUSSION

A marked negative change in Porphyridium at 703 mμ, originally interpreted as decreased absorption, is shown in Fig. 2. Waveform A was observed with actinic light only, while Waveform B was obtained by flashing the sample, with the monochromator set at 703 mμ. A spectrum of the changes between 650 and 850 mμ, obtained

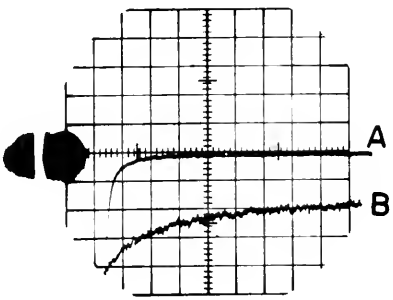

Fig. 2. Oscilloscope display of the 703 mμ change in Porphyridium. 10 mv. AC per vertical division; 200 μsec. per horizontal division. Waveform A: flash only. Waveform B: flash plus 703 mμ measuring light of 2 v. transmitted signal.

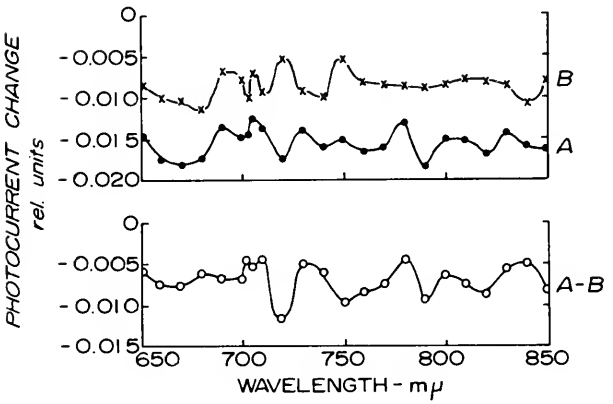

Fig. 3. Spectra of the negative changes in live and killed Porphyridium. Two pictures of flash plus measuring light and one flash-only picture were taken at each wavelength. Curve A: live Porphyridium. Curve B: killed Porphyridium.

with Porphyridium in 0.1 M $NaHCO_3$, is shown in Fig. 3, A. The sample was excited with light transmitted by a Corning 4-72 filter (335 - 630 mμ). Several shallow maxima can be observed, but no predominant change in the vicinity of 700 mμ (cf. Ref. 3, p. 324). The half-life of the change at 703 mμ was found to be 1.6 milliseconds.

The spectrum was then obtained with killed Porphyridium, in order to evaluate its suitability as a blank. An aliquot of the same sample used to obtain Spectrum A of Fig. 3 was placed in a boiling water bath. During the first 40 minutes at 100°, the magnitude of the 703 mμ change remained essentially the same as in live Porphyridium, which suggests that the change may be non-enzymatic in live Porphyridium as well. After two hours in the water bath, Porphyridium maintained about one half of its initial activity. The spectrum in killed Porphyridium (two hours at 100°) adjusted to essentially the same absorbance as live Porphyridium at 703 mμ is presented in Fig. 3, B; A - B, the difference between the spectra for live and killed Porphyridium, is also shown. The above spectra, if interpreted as absorption changes, would suggest a non-specific, purely photochemical bleaching,

possibly due to excessive flash intensity.

The possibility of instrumental artifacts was investigated by comparing the waveforms, with and without measuring light, of several non-photochemical systems, such as a $CaCO_3$ suspension and nutrient medium. The data obtained indicated the absence of such artifacts.

An extended spectrum of the changes in Porphyridium (Fig. 4) was obtained by exciting the sample, in nutrient medium, with light absorbed primarily by chlorophyll a (Corning Filter 5-58, transmitting between 350 and 480 mμ). Greater reproducibility was attained by taking 4 pictures with the flash plus measuring light on, and 2 flash-only pictures, at each wavelength. The effect of reducing the range of the exciting light was a nearly threefold reduction in the magnitude of the change at 700 mμ, partially attributable to a considerable reduction in integrated flash intensity. Several maxima are apparent in Fig. 4, including one at 700 mμ.

Since the magnitude of the change at 700 mμ was approximately 5 times that reported by Kok and Hoch (4), we investigated the possibility that the negative change observed was partially attributable to increased emission (rather than transmission) during and following the flash. To this end, we successively placed various interference filters in front of the phototube, setting

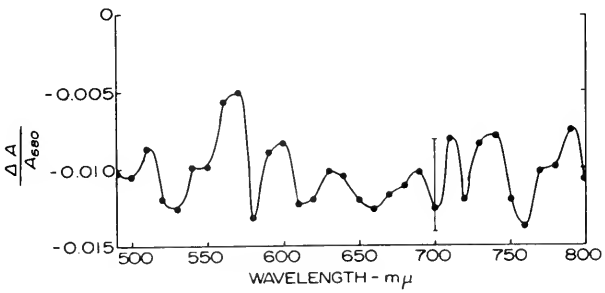

Fig. 4. Extended spectrum of the changes in Porphyridium, with chlorophyll excitation. The vertical line at 700 mμ represents the range of several means at that wavelength. ΔA: apparent change in absorbance. A_{680}: absorbance at 680 mμ.

the monochromator at the same wavelength as the transmission maximum of a given filter, while maintaining the same intensity of monochromatic light reaching the phototube as in the absence of the interference filter.

The results presented in Table I demonstrate that most of the increment in light intensity detected by the phototube following a flash is of a wavelength range other than that of the monochromatic beam. Hence, it is evident that the negative changes observed represent primarily fast, reversible increases in luminescence. The difference between the total change and the change observed with the interference filter in the path of the monochromatic light evidently provides a minimum estimate of the emission change; the fraction of luminescence transmitted by a given filter (or a monochromator located between the sample and the phototube) would still act as a negative absorption change, superimposed on a true absorption change of the same or opposite sign.

TABLE I
CONTRIBUTION OF EMISSION CHANGES TO THE NEGATIVE CHANGES OBSERVED IN PORPHYRIDIUM

	$\Delta A/A_{680}$ [1,2]		
Wavelength mμ	Total change	With interference filter	Minimum percent accounted by emission
550	-0.0089	+0.0012	100
680	-0.0041	-0.0018	56
730	-0.0103	+0.0003	100

[1] ΔA: apparent change in absorbance. A_{680}: absorbance at 680 mμ.

[2] Four pictures of flash plus measuring light and two flash-only pictures were used to obtain each value.

Next, the nature of the change at 700 mμ was investigated in Chlorella and spinach chloroplasts, in which the change was reported by Kok (3), as well as in aged chloroplasts with reduced phenazine methosulfate (PMS) studied by Witt et al. (7). The findings at 700 mμ (Table II) were similar to those for Porphyridium at other wavelengths.

TABLE II
CONTRIBUTION OF EMISSION CHANGES TO THE NEGATIVE CHANGES OBSERVED AT 700 mμ

System	$\Delta A/A_{680}$ [1,2]		
	Total change	With interference filter	Minimum percentage accounted by emission
Chlorella	-0.0055	-0.0001	98
Spinach chloroplasts	-0.0054	+0.0014	100
Spinach chloroplasts aged for 5 days, 5×10^{-6} M PMS and 5×10^{-5} M ascorbic acid	-0.0071	-0.0008	89

[1] ΔA: apparent change in absorbance at 700 mμ. A_{680}: absorbance at 680 mμ.

[2] Three to four pictures of flash plus measuring light and 3-4 pictures of flash only per value.

While the data presented demonstrate that interference from luminescence can be considerable, it certainly does not follow that emission changes interfered to the same extent in the work of other investigators. The experimental conditions that were suitable for observing the 703 mμ change in our apparatus differed in several respects from those selected by other workers. For example, our flash intensities were probably of a higher order of magnitude than Kok's.

Several procedures for circumventing the interference of luminescence were considered. Locating the monochromator between the sample and the phototube (cf. Fig. 1) would still involve the uncertainty that at some wavelengths an emission change would be superimposed on the absorption changes.

The essential features of an apparatus design (Fig. 5) and a procedure for resolving the contribution of emission and absorption changes to overall changes in light intensity are given below. Two identical red-sensitive phototubes, P_1 and P_2, are located symmetrically with respect to the flashtube, so that the flash-only waveform will appear the same. P_2, located at right

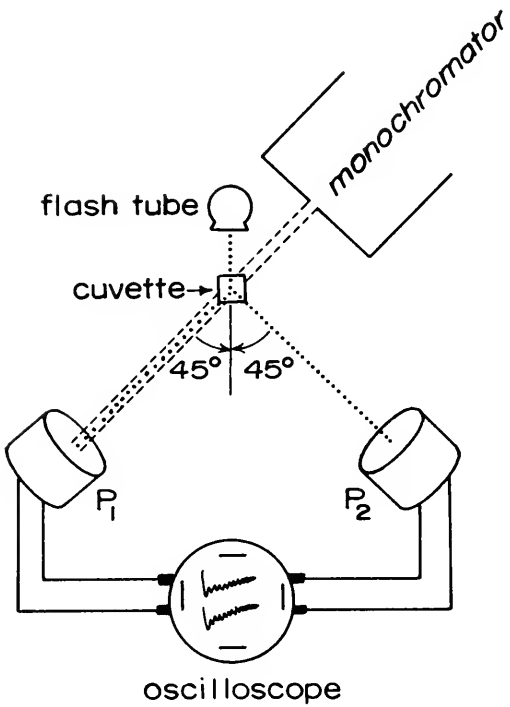

Fig. 5. Experimental arrangement for resolving the contribution of luminescence and absorption changes to fast changes in light intensity. Lenses, filters, and baffles are not shown.

angle to the monochromatic beam, detects changes in emission superimposed on the flash-only waveform, while the waveform of P_1 consists of three components, the absorption change, the emission change, and the flash intensity. The two waveforms are displayed simultaneously on the screen of a multichannel oscilloscope, while the absorption change is the difference waveform obtained directly by connecting the output of the two phototubes tc a balanced oscilloscope input. A more precise estimate of the absorption change is obtained by placing identical interference filters, corresponding in transmission to the monochromatic beam, in front of P_1 and P_2. P_2 can also be used for studying the nature of the flash-induced luminescence by locating a monochromator

between it and the sample.

The assumption that the two phototubes will detect essentially the same emission change may be checked by means of the following procedure:

Let

ΔT = total change at time t (corrected for flash-only change)

ΔF = change observed with interference filter at time t (corrected for flash-only change)

ΔE = contribution of the emission change to ΔT

Δe = contribution of the emission change to ΔF

Δa = absorption change, assumed to be essentially the same in ΔT and ΔF

$\Delta E'$ = emission change at time t, measured independently (by P_2)

$\Delta e'$ = emission change with interference filter at time t, measured independently (by P_2).

By definition

$$\Delta T = \Delta E + \Delta a \tag{1}$$

$$\Delta F = \Delta e + \Delta a \tag{2}$$

Assuming that

$$\frac{\Delta e}{\Delta E} \sim \frac{\Delta e'}{\Delta E'} = k \tag{3}$$

and solving Equations 1, 2, and 3 simultaneously, we have

$$\Delta a = \frac{\Delta F - k\Delta T}{1 - k} \tag{4}$$

Values of Δa calculated from Equation 4 can be compared with values obtained directly by subtracting the waveforms of the two phototubes. This method could also be used to estimate the magnitude of absorption changes in an apparatus where emission changes can be measured independently but direct corrections for emission cannot be made, because of differences in phototube geometry.

Understanding this flash-induced luminescence may prove of greater value than recognizing it as a possible experimental pitfall. It is analogous in some respects to a chromatic transient effect. Apparently, this luminescence represents either afterglow or fluorescence. Since the monochromatic beam is well below the compensation point in intensity, it is difficult to accept that it could have such a large effect on the flash-induced afterglow, which is feeble to begin with (as indicated by Fig. 2, A). Thus, we tentatively identified this effect as a flash-induced increase in the fluorescence originating from the detecting beam.

Such large rapid increases in fluorescence yield that could account for these emission changes have not been reported, to our knowledge; on the other hand, cases of large increases in fluorescence during prolonged bleaching are well known (1). By analogy to the steady-state situations, we visualize the flash as bringing about a temporary bleaching of some chlorophyll, either by photooxidation or by photoionization. This process would temporarily impede the flow of excitation energy through the photosynthetic units, resulting in increased dissipation as fluorescence.

SUMMARY

Relatively large changes in emission may be superimposed on changes in absorption when observations are made with a red-sensitive flash apparatus. Accordingly, it is desirable to reexamine some of the findings on the 703 mμ change. An apparatus design and a procedure for resolving the contribution of absorption and emission changes to overall changes observed with a flash-apparatus are described. As a tentative mechanism for flash-induced luminescence, we propose that the actinic light by bleaching some chlorophyll causes a temporary disruption of energy migration with a concomitant temporary increase in fluorescence.

ACKNOWLEDGEMENT

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EFFECTS OF HYDROSTATIC PRESSURE ON INDUCTION TRANSIENTS OF OXYGEN EVOLUTION

William Vidaver

SUMMARY

A transient oxygen evolution spike was seen at about the first second of illumination in the induction period of some species of brown and green algae. The transient normally occurred in Ankistrodesmus, but required abnormally high or low temperatures in Ulva and Ilea. It never appeared in Porphyra. Hydrostatic pressure high enough to generally reduce subsequent oxygen evolution rates scarcely lowered the very rapid transient rates.

Lack of pressure sensitivity suggests that the transient is due to a non-enzymatic system which produces oxygen in the light. It appears that unless the products of this system are consumed as they are produced, steady rate oxygen production does not occur.

INTRODUCTION

Polarographic detection shows a complex time course of oxygen evolution for many algae as a response to illumination. With a possible exception, the induction responses of algae are generally similar. Usually only the relative magnitude of time course response components differs between various kinds of algae.

Figure 1 represents a recording of the induction time course of oxygen evolution upon exposure of the marine alga Porphyra perforata to white light of approximately one-half saturation intensity. The relatively rapid rise to a peak (designated the a spike) was followed by a downslope (b) and a slow rise (c) and eventually culminated in a more or less steady rate under continued illumination. Some algae display a distinct pre-a transient during the spike rise[1, 7].

The parameter of varied hydrostatic pressure has been useful in elucidating kinetic data pertaining to rates of some biological and biochemical reactions[2, 3, 5]. In photosynthetic plants, oxygen evolution can also be observed under changing physical conditions, including variation of hydrostatic pressure. Hydrostatic pressure applied to photosynthesizing algae alters the induction

time course of oxygen evolution during illumination.

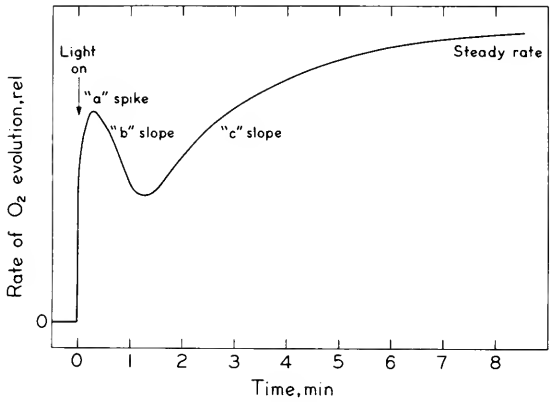

Fig. 1. Induction time course of oxygen evolution in Porphyra perforata. (15° C., 1000 psi.)

In this study two types of observations of pressure effects were made on the time course of oxygen evolution induction in algae. One of these recorded oxygen evolution rates under constant illumination; the other determined pressure influence on oxygen evolved by algae in response to light flashes.

Four algal species were observed in these experiments. These were selected because they represent some of the wide variety of algal types. A second consideration of selection was that the marine species are extremely thin thalloid forms and consequently present minimal barriers to light transmission and gas diffusion. Porphyra perforata is a marine red alga one cell thick, while the green marine alga Ulva lobata consists of two cell layers. Ilea fascia, a marine brown alga, has a complex parenchymatous thallus, but is quite thin in cross section. The green freshwater unicellular alga, Ankistrodesmus falcatus, was also used.

METHODS

Discs of algal thalli were placed in contact with a platinum electrode polarized at -0.6 v., which along with a silver-silver chloride reference electrode, could be enclosed in a pressurized optical cell (described elsewhere)[7]. Unicellular algae were taken up on a disc of cigarette paper which was then applied to the electrode. For marine algae, seawater was used as both the medium and the hydraulic fluid. Tapwater was substituted for the

freshwater algae. CO_2 concentration was 4 per cent by volume at the start of the experiment. O_2 was assumed to be in equilibrium with the atmosphere. Light was projected in a collimated beam from a 100w tungsten incandescent lamp and focused on the algal disc in the pressurized cell.

The intensity of white light was adjusted to bring about a steady oxygen evolution of approximately one-half the saturation rate at the base pressure of 1000 pounds per square inch (psi) for the experiments with constant illumination. Pressure of this magnitude has an imperceptible effect on oxygen evolution and ensures that gasses (CO_2 and O_2), in the experimental concentrations, remain dissolved in the medium.

For the light flashes, intensity was empirically selected to induce maximum transient response and was usually near saturation (at 1000 psi).

RESULTS

Constant Light

Time courses of the three thalloid algae show that both steady oxygen evolution and the a spike were inhibited by increased hydrostatic pressure (Figure 2a, b, c). There was greater inhibition of steady rates than of the highest rate of the spike.

Ankistrodesmus (Figure 2d) was affected similarly by pressure. However this alga displayed a small transient peak which occurred before the maximum of the a spike. The transient appeared to be almost uninhibited by the highest applied pressure.

Light Flashes

In view of the pre-a transient observed in the induction period of Ankistrodesmus, attempts were made to ascertain the possibility of a pre-a transient being present in the induction response of other algae. It was found that high or low temperature (4° or 30° C.) did induce the appearance of similar transients in both Ulva and Ilea. No pre-a transient was ever observed in the response of Porphyra. Figures 3 and 4 show that the pre-a transients of Ulva and Ilea are also strikingly resistant to pressure inhibition.

DISCUSSION

A pressure insensitive oxygen evolution transient was observed

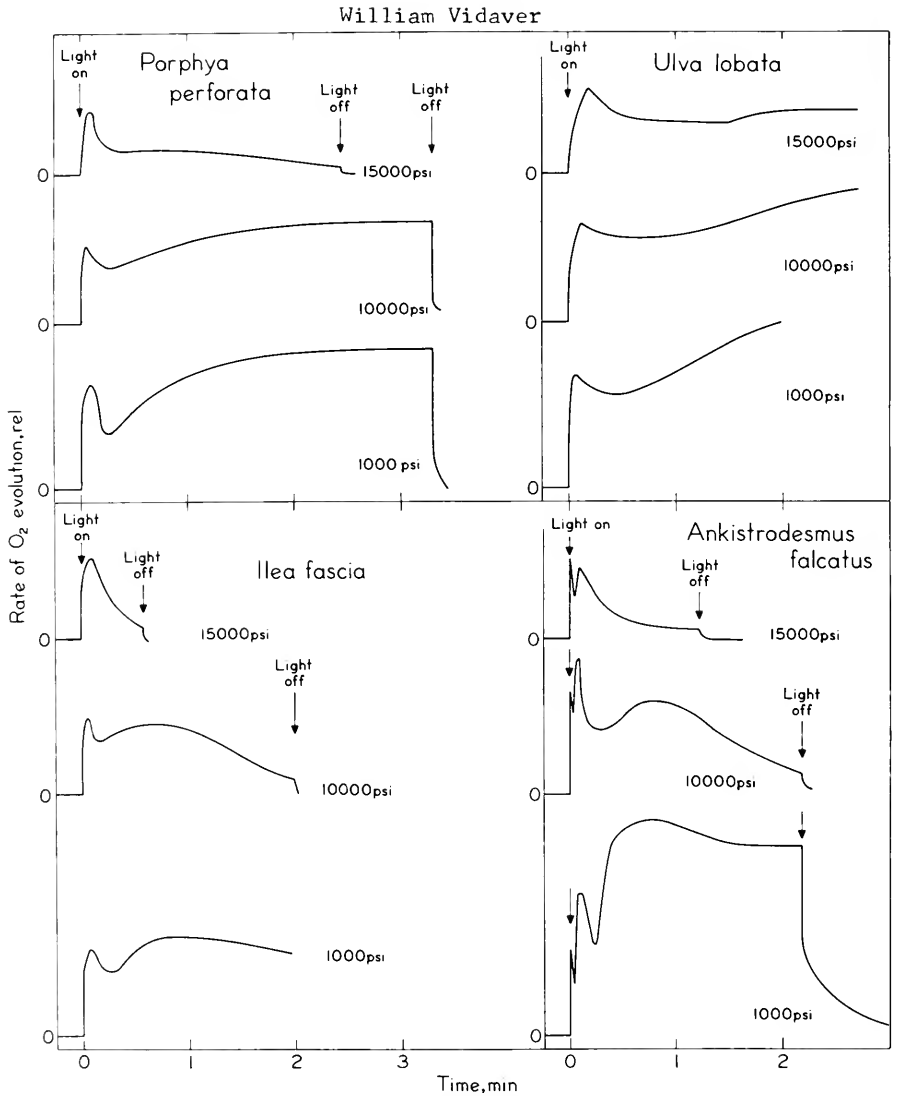

Fig. 2. Induction time courses of oxygen evolution at three different hydrostatic pressures. Intensity of white light was approximately one-half that required for steady rate saturation. No pre-a transient (described in text) is visible in the response of the marine algae (a,b,c). A distinct transient appears on the rise of the a spike in Ankistrodesmus (d). While steady rates were affected more by pressure than maximum a spike rates, the pre-a transient is least inhibited (15°).

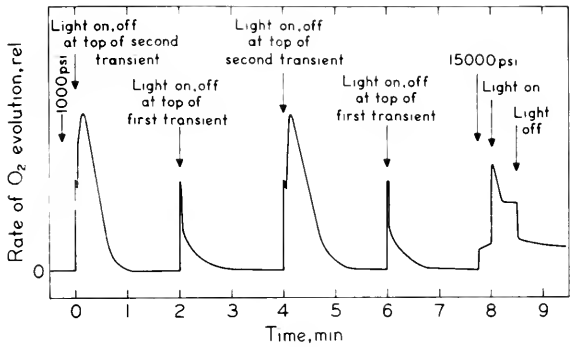

Fig. 3. Isolation of the pre-a transient in Ulva by low temperature (4° C.) and light flashes. Transient magnitude is almost undiminished by the highest applied hydrostatic pressure (15000 psi).

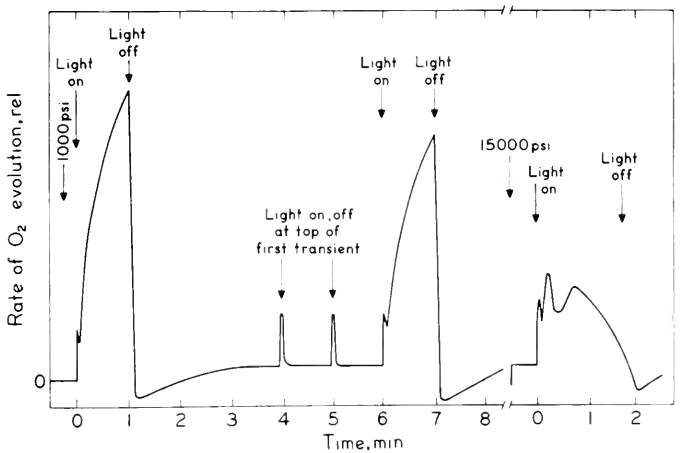

Fig. 4. Pre-a transient isolation in Ilea at high temperature (30°). The transient persists at the highest applied pressure.

in the induction period during illumination in species of brown and green marine algae and a freshwater green alga. This transient could not be observed in the response of the red alga Porphyra.

In those algae displaying the transient, oxygen must be evolved as a response to light by a mechanism which is presumably insensitive to inhibition by hydrostatic pressure of at least 15000 psi. This response appears to differ kinetically from those usually associated with enzymatic reactions[6, chap. 9]. Variations of temperature and pressure seem to have a greater effect on the rates of those processes which proceed more slowly than the reactions involved with the transient. By further slowing through temperature extremes or the application of hydrostatic pressure, it is possible to isolate (by the elimination of swamping) the pre-a transient in algae that normally do not show it.

The transient may result from the functioning of a primary oxygen-evolving mechanism, since its lack of pressure sensitivity suggests that it is not the result of enzymatic reactions. If this response is due to a photolytic water reaction, then it may be presumed that oxygen evolution in plants that show the transient is the result of rapid photochemical reactions, and does not require the intervention of enzymatic transport systems. If it is assumed that a very early response to light is the evolution of oxygen, and that oxygen evolution does not continue unless the products formed during this process are consumed in some way (Hill reaction), then it may be expected that only the transient would appear in response to illumination. Hydrostatic pressure blocks the reactions by which the photoproducts seem to be utilized. Consequently, the transient persists under pressure, while steady rate oxygen evolution is inhibited. This response is analogous to that of isolated chloroplasts lacking an added Hill oxidant which show only a single, transient oxygen-production spike. These chloroplasts require a certain dark interval before the maximum transient rate may be regained[4]. Experiments not presented here indicate a similar, though shorter time course for maximum transient recovery in whole cells.

The high or low temperature requirement for induction of the transient in some algae may result from rate changes in enzymatic reactions that utilize the products of the oxygen-evolving photoreactions. Reaction rate theory[6, chap. 8] predicts inhibition of enzymatic reactions as a consequence of temperature extremes. Low or high temperature could induce the appearance of the transient by the reduction of the relatively high rates of the reactions which consume the immediate photoproducts (other than molecular oxygen).

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The apparent lack of the pre-a transient in Porphyra may be real and therefore suggests a process of oxygen evolution different from that of green or brown algae. On the other hand it is possible that the experimental treatment did not succeed in isolating the transient from the rest of the induction response.

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EFFECTS OF PHOTODYNAMIC TREATMENT, ULTRAVIOLET RADIATION AND GAMMA RADIATION ON THE PHOTOSYNTHESIS AND HILL REACTION OF CHLORELLA

John D. Spikes and Dennis C. Hall

Our research program has been concerned primarily with kinetic studies of the ferricyanide and quinone Hill reaction of isolated chloroplasts of higher plants[1,2]. In addition, we have done some comparative work on the photosynthesis and whole-cell quinone Hill reaction of Chlorella[3]. We have been especially interested in mechanisms of energy absorption, transfer and utilization in chloroplast systems as well as in the water-splitting process of the Hill reaction[4]. More recently we have expanded our program to include a somewhat simpler photochemical phenomenon, photodynamic action. Photodynamic action may be defined operationally as the killing or chemical alteration of an organism, cell, or virus. or the chemical alteration of a molecule by light in the presence of molecular oxygen and an appropriate photosensitizing dye[5,6]. In this process, light energy is absorbed by the sensitizing dye and then transferred in some manner to the substrate system.

In a sense, energy transfer mechanisms in photosynthesis and in photodynamic action may be considered analogous, since long-lived, light-excited states of the sensitizing pigments are apparently involved in both cases. If energy transfer from excited chlorophyll in a cell is prevented, the energy will be used in reactions inhibitory or destructive to the photosynthetic apparatus. In many cases these destructive reactions are photodynamic in nature (see [7] and [8] for reviews of this field). Photosynthetic cells lacking carotenoids are rapidly destroyed under aerobic conditions in the light in what is apparently a chlorophyll-sensitized photodynamic process[8].

This paper is concerned primarily with studies on the photodynamic inactivation of the photosynthesis and whole-cell quinone Hill reaction of Chlorella in the presence of added dyes. For comparative purposes, some data on the inactivation of these same

processes by 2537 Å ultraviolet radiation and by Cobalt-60 gamma radiation is also presented.

Considerable work has been done on the differential staining of plant cell organelles with various dyes. Some dyes, such as Rhodamin B, appear to localize in the chloroplasts[9]. Staining Elodea leaves in the light decreased photosynthesis; staining in the dark had no effect[10,11]. Similarly, chloroplasts of cells stained in the light showed a decreased ability to reduce infiltrated silver nitrate[12].

Space prevents a detailed discussion of the literature relating to effects of ultraviolet and ionizing radiation on photosynthesis and the Hill reaction. These fields have been recently reviewed, however[7,8]. Some work has been done on the comparative kinetics of ultraviolet inactivation of the different photochemical processes carried out by algae. In Scenedesmus, for example, photosynthesis, the Hill reaction (as measured using an indophenol dye), and photoreduction were all inhibited about equally in a first-order manner by 2537 Å ultraviolet radiation[13]. No comparable comparative studies with ionizing radiation appear to have been published. The photosynthesis of Chlorella as measured by carbon dioxide fixation and oxygen evolution can be partially inhibited by gamma irradiation[14]. More recently, considerable work has been done on the effects of ionizing radiation on the photochemical properties of isolated chloroplasts (see [7,8,15] for references).

MATERIALS AND METHODS

The experiments were carried out with Chlorella pyrenoidosa, Chick, Emerson strain, type D, grown at 25°C. in a modified Knopp medium with continuous agitation while being bubbled with 3% carbon dioxide in air. The algae were grown for 72 hours under continuous illumination (800 foot-candles of "white" fluorescent light) and then for 84 hours under alternate 12 hour light and dark periods. Measurements of photosynthesis and Hill reaction were made manometrically at 15°C. using rectangular glass vessels. The vessels were illuminated with 150-watt reflector-flood bulbs; light intensity was approximately 4000 foot-candles. The Hill reaction system was 0.004M in p-benzoquinone, 0.05M in pH 6.8 sodium phosphate buffer, 0.01M in potassium chloride and contained approximately 1% algae, all in a total volume of 3 ml. Photosynthesis was measured using Warburg No. 9 buffer.

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Photodynamic action systems contained 1.0 x 10^{-4} M dye. Illumination with shaking in contact with air was carried out with the same apparatus used for manometric measurements. After treatment, the algae were washed several times in distilled water and then resuspended in distilled water. In the routine screening of dyes for photodynamic activity, algal suspensions containing dye were incubated in the dark at 15°C. for one hour. Samples were then illuminated for one hour. "Dark" and "light" samples were washed several times and then assayed for photosynthetic and Hill reaction activity. Appropriate light and dark controls were also carried out.

A General Electric 15-watt germicidal lamp was used in the ultraviolet radiation studies. Ultraviolet radiation intensities were measured with a Luckiesh-Taylor "germicidal attachment" in conjunction with a General Electric Model 80W40V16 light meter. Gamma radiation studies were carried out with a 525 curie cobalt-60 source. The irradiation chamber was maintained at a constant temperature. The energy flux in the chamber was measured periodically with the Fricke ferrous sulfate method; the dose rate was 73.1 $\pm$ 0.3 x 10^3 rads per hour.

RESULTS

Photodynamic effects on Hill reaction and photosynthesis-

A large number of dyes were examined for "dark" and photodynamic effects on Chlorella Hill reaction and photosynthesis. Most of the dyes showed no photodynamic sensitization. The inactive dyes included Acridine orange, Acriflavine, Alcian blue, Auramine O, Aureomycin, Azure A, Azure C, Biofluor, Camoquin dihydrychloride, Dinitrophenol, Eosin B, Eosin Y, Fuchsins, Hematoporphyrin, Janus green B, Malachite green, 8-Methoxypsoralen, Neutral red, Nile blue A, Orange G, para-Rosanilin hydrochloride, Pentaquine phosphate, Phenosafranin, Pyronin B, Pyronin Y, Rhodamine B, Rhodamine G, Riboflavin, Riboflavin-5'-phosphate, Safranin O, Sodium magnesium chlorophyllin, Toluidine blue O, Toluylene blue, and Trypan blue. Seven of the dyes were photodynamically active, including Erythrosin B, Ethyl eosin, Methylene blue, New methylene blue N, Phenazine methosulfate, Rose bengal, and Thionin. Some dyes, including Malachite green, Methylene blue, Nile blue A and Rose bengal produced inactivation in the dark at higher concentrations. Photosynthesis was usually more sensitive to dark inactivation than the Hill reaction.

The dye, Rose bengal, which combined good photosensitizing

ability with average dark activity was selected for more detailed studies. Photochemical inactivation with Rose bengal followed the classical photodynamic pattern in that systems illuminated under flowing tank nitrogen showed only slight loss of Hill reaction and photosynthetic activities, while systems illuminated in the same way in air showed a rapid loss of activity. Inactivation was apparently not produced to any great extent by stable toxic photoproducts, since samples of Rose bengal which had been illuminated in air were no more inhibitory in the dark than unilluminated dye.

The dark and light inactivation of Chlorella in the presence of Rose bengal was investigated as a function of various combinations of dye concentration, duration of dark treatment with the dye, and duration of illumination in the presence of the dye. Treatment with dye in the dark inhibited photosynthetic activity; inhibition increased with the duration of treatment. Hill reaction activity was relatively insensitive to dark treatment. Inhibition of both activities increased progressively with duration of illumination in the presence of dye. Loss of activity in the light was not strictly first-order. Inactivation, in general, increased with increasing dye concentration. A typical set of data illustrating the effects of Rose bengal concentration on both dark and light inactivation is shown in Table I.

	LIGHT + DYE			DARK + DYE			UNTREATED
DYE MOLARITY	10^{-6} M	10^{-5} M	10^{-4} M	10^{-6} M	10^{-5} M	10^{-4} M	
HILL REACTION (mm^3 O_2/ HR)	302	212	29	373	359	354	378
PHOTO-SYNTHESIS (mm^3 O_2/ HR)	260	129	-56	337	282	73	347

Table I - Effect of Rose bengal concentration on the photodynamic and dark inactivation of Chlorella photosynthesis and quinone Hill reaction. The algae were treated in the dark with dye in the concentrations given for 60 min., and then illuminated for 15 min. at 4,000 foot-candles in the case of the light experiments.

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As may be seen, the Hill reaction activity fell off progressively with increasing dye concentration in the photodynamic experiments, whereas little inactivation occurred if the algae were kept in the dark. In the case of photosynthesis, activity fell off rapidly with increasing dye concentration in both the light and in the dark; activity was lost more rapidly in the light. The algae treated photodynamically in the presence of high concentrations of dye actually took up oxygen on illumination in the photosynthetic system.

Ultraviolet effects on Hill reaction and photosynthesis-

Space permits the presentation of only a few observations on the comparative effects of 2537 Å ultraviolet radiation on algal Hill reaction and photosynthesis. When the algae were irradiated at a dose rate of 1360 microwatts/cm^2, the photosynthetic rate dropped to 50% after 3 min. and to zero after 5 min. In contrast, the Hill reaction rate increased sharply at first and attained a value 21% greater than that of the control after one min. of irradiation. The activity then decreased rapidly with dose, and dropped to 50% after 4 min. and to zero at 5 min. After the transitory initial rise, the time-course for loss of Hill reaction activity closely resembled that for the loss of photosynthetic activity. In neither case did the loss of activity follow first-order kinetics. Algae irradiated for 6 min. or longer took up oxygen on illumination at a rate 15-20 times greater than the rate of uptake in the dark. No photoreactivation of the Hill reaction or photosynthetic activity in ultraviolet-irradiated algae was observed.

Gamma radiation effects on Hill reaction and photosynthesis-

Again, space permits the presentation of only a few highlights in this area of work. The loss of both Hill reaction and photosynthetic activities was approximately linear with gamma radiation dose. Loss of activity was greater at higher irradiation temperatures; the loss of Hill reaction activity was more temperature-dependent than the loss of photosynthetic activity. At 12°C. the Hill reaction fell off more rapidly with increasing dose (50% inactivation at 550,000 rad) than did photosynthetic activity (50% inactivation at 790,000 rad).

Cyanide protected algal Hill reaction significantly from gamma radiation damage, while beta-aminoethylisothiuronium hydrobromide (AET) had no protective effect. Both light and oxygen protected

Chlorella during gamma irradiation, with Hill reaction activity being protected to a slightly greater extent than photosynthetic activity.

Chlorophyll destruction in gamma-irradiated algae was relatively very much less than the inactivation of Hill reaction and photosynthetic activities. Therefore decrease in activity could not be correlated in any simple way with the loss of bulk chlorophyll. The extractability of chlorophyll from the algae into 95% methanol was increased by gamma irradiation. Chlorophyll in solution in 95% methanol was much more sensitive to destruction by gamma radiation than chlorophyll in situ in the algal cell.

The Hill reaction and photosynthetic activities of both ultraviolet and gamma-irradiated algae were retained during post-irradiation storage in much the same way as in unirradiated algae. Activities were lost rapidly during storage in the dark at higher temperatures, and slowly in the light at low temperatures. There was no evidence of photoreactivation in gamma-irradiated algae.

DISCUSSION

The present work demonstrates the photodynamic inactivation of Chlorella photosynthesis and Hill reaction in the presence of certain dyes. These dyes presumably penetrate through the cell membrane and into the chloroplast in order to act as sensitizers. The apparent lack of activity of dyes such as Rhodamine B, which have been shown to photosensitize other systems, may thus result from slow penetration into Chlorella cells rather than from lack of activity. Some dyes inhibited photosynthetic activity by dark reactions; Hill reaction activity was much less sensitive. Presumably this inhibition results from the ability of these dyes to associate or react with cellular constituents involved in carbon dioxide-fixation pathways. Such dyes might be useful specific inhibitors of particular steps in these pathways.

The study of photodynamic processes may be justified in the context of photosynthetic mechanism from several points of view. First, a number of workers have demonstrated that chlorophyll-sensitized endogenous photodynamic processes occur in photosynthetic plants under certain conditions. Plants therefore survive exposure to light only by possessing devices to minimize photodynamic action. Secondly, there may be useful analogies between simple photodynamic systems and photosynthesis which

would aid in understanding the latter process. This follows since both systems absorb light in approximately the same energy range and then transfer this energy to other molecules through the mediation of some long-lived state of the pigment. Finally, photodynamic action might be used as a specific inhibitor in studying sub-reactions of photosynthesis. Chlorella photosynthesis is more sensitive than the Hill reaction to photodynamic inactivation in the presence of Rose bengal. Different dyes, because of different chemical and physical properties, might be expected to localize at different sites in the photosynthetic reaction sequences. On subsequent illumination, different sites could then be preferentially inactivated. As an example of this approach, guanine residues are found to be destroyed preferentially when DNA is illuminated in the presence of methylene blue; the other bases are little affected[16].

In contrast to earlier studies[13], our data on the time course of inactivation of Chlorella Hill reaction and photosynthesis by ultraviolet radiation did not fit first-order kinetics. The dose required for 50% inhibition of photosynthesis fell within the range previously observed[17]. It is not clear from our data whether the ultraviolet-inactivation of Hill reaction and photosynthetic activities involves the same "sensitive" molecule. With low radiation doses, Hill reaction activity is stimulated significantly while photosynthetic activity immediately decreases. However, after the initial stimulation, Hill reaction activity decreases with increasing dose in much the same way as photosynthetic activity.

Packard[18] reported that photosynthetic organisms were less sensitive to damage by ionizing radiation when irradiated in the light. The present work shows that Chlorella cells illuminated during gamma irradiation retained more than twice as much Hill reaction and photosynthetic activity as algae irradiated with the same dose in the dark. Another interesting observation was that oxygen partially protected these activities against inactivation by gamma radiation. Such a "reverse" oxygen effect has been observed only rarely[19]. Chlorophyll *in situ* was much less sensitive to destruction by gamma radiation than chlorophyll dissolved in organic solvents (also see [20]). It may be that certain constituents of the chloroplast protect the pigment in some way, or the pigment may be present in the chloroplast in a physical form (such as micro-crystals) much more resistant to ionizing radiation than chlorophyll molecules in solution. The increase in extractability of chlorophyll from Chlorella with

increasing dose of gamma irradiation is similar to that reported for ultraviolet irradiated leaves(21).

Algae in which photosynthesis was inactivated by photodynamic treatment, or by acute doses of ultraviolet or gamma radiation, showed a large uptake of oxygen on illumination. The rate of uptake was many-fold greater than the respiratory rate. In inactivated algae the chlorophyll excitation energy probably cannot be channeled into useful reactions and is therefore available for the photooxidation of endogenous organic compounds.

In summary, we would like to suggest again the use of radiation as a tool of the "specific inhibitor" type in the elucidation of photosynthetic mechanisms. The present experiments, although very preliminary, do demonstrate the differential effects of radiation on the photosynthetic and Hill reaction activities of Chlorella. Because of the possibility of using selective dyes, the photodynamic inactivation approach appears to be the most useful.

ACKNOWLEDGMENTS

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ON THE VARIABILITY IN THE ACTIVITY OF THE PHOTOSYNTHETIC MECHANISMS

Constantine Sorokin

Photosynthetic activity was observed to vary from one observation to another and in the course of one experiment. For example, in measurements of the enhancement effect, the photosynthetic rate and enhancement were changing over a considerable period of time estimated to last for the red alga Porphyridium for about 4 hours [1]. In Chlorella, the dark current was observed to drift during the first 12 hours after placing cells upon the electrode [2], and stabilization of photosynthetic oxygen production required up to 2 or 3 days. Poor reproducibility and inconsistency of the activity with time, even for the same batch of cells, implied that some factor or factors were possibly overlooked.

Studies on photosynthetic activity in the course of the development of algal cells indicated that the activity level does not remain constant during the life cycle of cells. As a cell proceeds in its development, the activity first rises during the initial portion of the life cycle, attains a maximum in cells of intermediate age, and then declines toward the time of cell division (Fig. 1).

One group of investigators observed the rising part of the curve to coincide with the initial portion of the illumination period and the declining part of the curve with the later portion of the illumination period [3-7]. Under conditions of synchronization used by another group of researchers, the recovery of the rate was accomplished before the beginning of illumination; thus, the decline in the photosynthetic rate was usually observed as extending over the whole light period [8, 9].

A decline in photosynthetic activity beginning at some earlier or later developmental stage is a common feature characteristic of the life cycle of cells. Old cells are sluggish [6], have lower quantum efficiency [10], and are more sensitive to adverse environmental conditions, particularly to the effects of strong light [6].

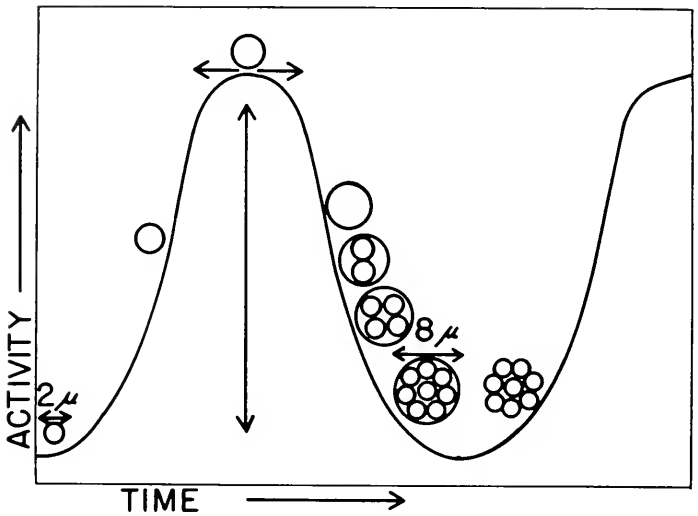

Fig. 1--Photosynthetic activity in the course of cell development.

In prolonged measurements, the rate of photosynthetic gas exchange declines in old cells from the very beginning of observations (Fig. 2). In younger cells, photosynthetic rate may greatly increase in the course of observations, even if cells are suspended in a nitrogen-free medium [11-14].

Thus, the size and/or activity of photosynthetic apparatus may increase in younger cells even under conditions generally considered unfavorable for photosynthesis. Under the same and more favorable conditions, the activity and/or the size of photosynthetic machinery in old cells inevitably declines.

The decline in the active part of photosynthetic apparatus with aging of cells has been attributed to changes in the rate of metabolic turnover: the increase in catabolic activity and the decline in intensity of anabolic processes characteristic of the process of cell development. An intense anabolic activity is coupled in younger cells with higher capacity to use metabolic intermediates for rebuilding photosynthetic machinery [16, 17].

Respiration activity [18] and the capacity of cells for

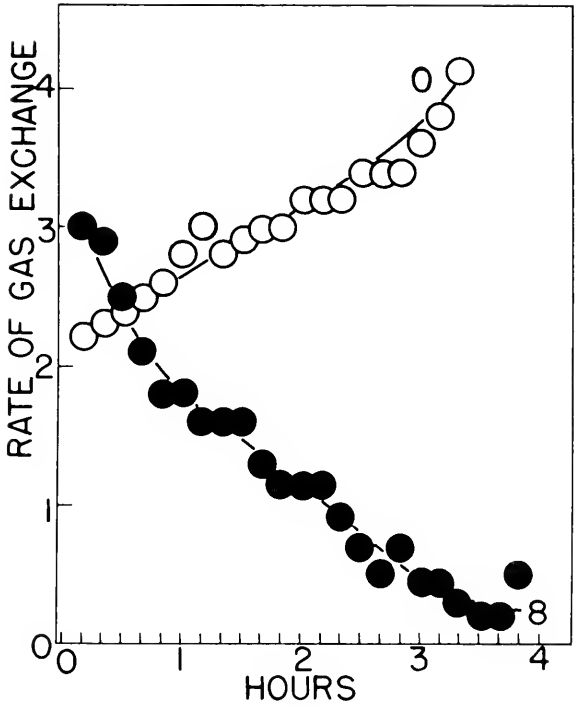

Fig. 2--Rates of photosynthetic gas exchange (in relative units) for 10-min. intervals in synchronized 0- and 8-hour cells of Chlorella [15].

growth [19] have been also reported to decline toward the end of the life cycle. Available information on nitrogen accumulation [20], nucleic acid content [21], ash constituents [22], phosphorylation activity [23], and contents of several vitamins [24] also indicates that all these variables depend on the developmental status of cells.

Surprisingly, observations on the dependence of metabolic activity on the developmental status of cells have made small impact on studies of metabolism, and particularly on studies of photosynthesis. A plausible explanation for the little consideration given to the developmental aspect of photosynthesis

lies, perhaps, with the fact that most of the above observations were made on specially synchronized algal cells.

There has been wide-spread concern [25-28] that the observed variations in metabolic activity may be due to synchronization technique; that they are not characteristic of the normal life cycle of cells but are caused by the synchronizing agent. The effect of a synchronizing agent on the subsequent activity of cells cannot be denied any more than can the effect of any other environmental condition. However, a decline in metabolic activity with the age of cells was observed also for the naturally synchronized green colonial alga Hydrodictyon [29] and for individual cells of Amoeba [30] and Tetrahymena [31].

Another technique used in studies of the relative photosynthetic activity in young and old algal cells consists of separation of cells of an originally nonsynchronized suspension into fractions by centrifugation. After centrifugation, the small cell fraction presumably consists mostly of young cells and the large cell fraction of predominantly old cells. Before being centrifuged from a nonsynchronized suspension, these two groups of cells are subjected to the same environmental conditions and the difference in their performance must, therefore, be attributed only to the difference in the age composition of these groups.

Using this technique, the late Dr. R. Emerson studied quantum efficiency of small and large cells and made two comments. In one report in 1954 [32], he stated that large cells have higher quantum requirement per molecule of photosynthetic oxygen production. In 1957 [33], he reversed his opinion, stating that difference in quantum efficiency of large and small cells is transient and therefore illusory. Neither report was published in detail, and no description of the technique used by Dr. Emerson is available. Dr. Emerson's observations, and particularly his denial of the age changes in photosynthetic activity, probably contributed to the delay in the acceptance of the concept of aging of cells as a universal biological reality.

Centrifugation technique may lead to false interpretations unless some basic conditions are observed. These conditions include:

1. A wide diversification in cell sizes of nonsynchronized suspension to be subjected to centrifugation;

2. A careful, repeated centrifugation and rejection of the largest portion of the intermediate cells;

3. A prevention of large cells from dividing during the process of centrifugation;

4. Prolonged observations on the activity of small and large cell fractions.

In our investigations under various temperature and illuminance conditions and in such diverse suspending fluids as phosphate buffer at pH 4.5, bicarbonate buffer at neutral pH, and carbonate-bicarbonate buffer at pH above 9, the fraction of larger cells consistently displayed an inferior photosynthetic activity provided the separation of cells into fractions by centrifugation was reasonably good (Fig. 3).

In studies on nonsynchronized algal populations a gradual, slow change in age composition of the batch of cells and an accumulation of small cells occur. Actual progress in cell division in a batch of cells under observation depends on the hereditary constitution of the organism, the external conditions, and the physiological state of the cells, the last one largely dependent on their prehistory. An interaction of these factors affects individual cells against their developmental background. Thus, the chance of a particular cell to enter the division stage, the degree of completion of cell division, and the time course of the division process depend on how far the cell progressed in its development by the time it was taken for observation. The complexity of the situation makes it unreasonable to expect a ready standardization of age composition of cells used in a series of experiments.

Eventually all cells capable of division divide and the age composition of the batch of cells stabilizes. These cells have been described as "active dark" cells [2]. However, for a number of reasons, cells maintained on the electrode surface hardly can be called "active dark" cells. For one reason, under unfavorable conditions, not all the cells divide and some cells do not attain the status of a small (dark) cell. For another reason, the "active dark" cells, in the sense used by Tamiya group [8], are recognized to be actually starved cells [34, 35]. Thirdly, physical conditions on the electrode surface are even less favorable for cell division and maintenance of cells than those stipulated by Tamiya's synchronization technique.

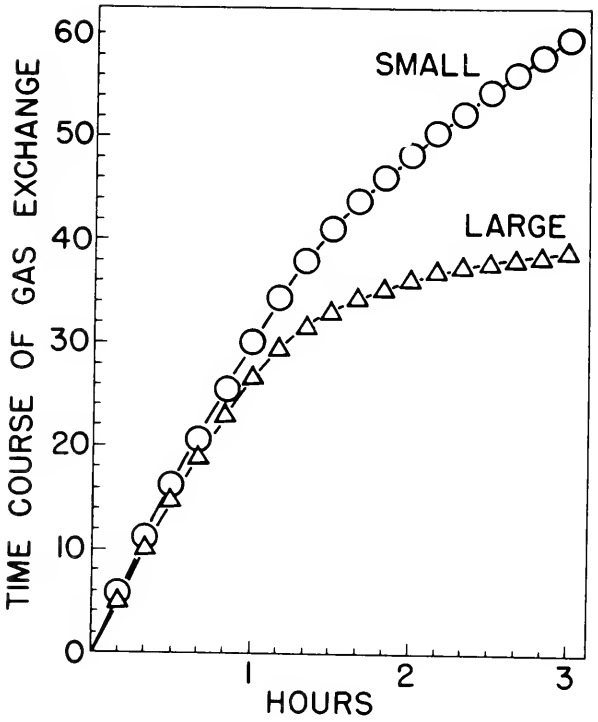

Fig. 3--Time course of photosynthetic gas exchange (in relative units) in small and large Chlorella cells separated from a nonsynchronized suspension by fractional centrifugation.

Growth under the described conditions [2] is negligible or absent. The importance of maintaining growth conditions has been generally disregarded in photosynthetic investigations. However, growth activity is a reliable indicator of the direction and rate of metabolic turnover. Catabolic activity is much stronger and anabolic processes are comparatively weak in nongrowing cells [36]. Enzyme proteins in nongrowing cells undergo fast degradation, products of degradation accumulate, and the size of the active part of the metabolic apparatus is reduced to a minimum.

The duration of the period of intensive degradation under unfavorable growth conditions is expected to depend on the physiological state of cells and on external factors. Eventually, with little left to undergo degradation, catabolic processes also decline to a minimum. In an impoverished cell, metabolic activity is more or less stabilized at a low level. However, the fact that the cell cannot be maintained in this state indefinitely indicates that even under these conditions degradation proceeds at a slow rate until the death of the cell.

Thus, in a batch of nonsynchronized cells undergoing the so-called starvation (2, 37), three groups of complex processes take place: the depletion of reserve materials in the process of respiration, a change in the age composition of cells under observation, and progressive degradation of metabolic mechanisms due to absence of growth. Both moieties of the photosynthetic apparatus, the one responsible for dark reactions and the other constituting photochemical part of photosynthetic machinery, are affected. An indirect evidence for the enzymatic part to be affected by age changes and metabolic turnover comes from studies on the degradation of enzyme proteins in bacteria and yeasts (36) as well as from observations on the decline in the respiratory and growth activity with the age of the cells (18, 19). A more direct proof is supplied by the fact that photosynthetic activity in older cells is much lower than in younger cells under conditions of light saturation where the rate of the photosynthetic process is affected by enzyme activity (6).

Indications for the photochemical part of the photosynthetic apparatus to be affected by changes bound with cell development and metabolic turnover are as follows:

1. Studies on chlorophyll turnover in higher plants indicated that chlorophyll undergoes a rapid degradation in older tissues (38-40).

2. Observations on synchronized algal suspensions demonstrated that photosynthetic activity in older cells is inferior to that of cells of intermediate age also at intensities below light saturation where the rate of photosynthesis is assumed to be dependent on photochemical reactions (41).

3. Fluorescence intensity for older cells was shown to exceed that of younger cells (42). With the inverse ratio existing between fluorescence yield and photosynthetic activity, observations on the increase in fluorescence with the age of the cells

provide most direct proof for changes in the distribution of energy in the primary photochemical act, changes bound with cell development.

Thus, a cell never stands still. It is in a constant flux. An interplay of biological factors may explain the inconsistency of observations on photosynthetic activity of cells. Of these factors, shifts in the age composition in a batch of cells under investigation and changes in the size of the active part of metabolic mechanism in each particular cell, as affected by metabolic turnover, deserve thoughtful consideration.

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SUMMARY

This summary is an attempt by the chairman and organizer to present a brief, moderately critical guide to major points of experimental and theoretical interest in the 70-odd reports collected in this volume. The reader should be aware, however, that the emphases and interpretations of this survey are unavoidably biased.

At the present meeting the general focus of attention was on the questions revolving around the cooperation of two distinct light reactions, and in particular, the electron transport pathways associated with each. Since many papers in this volume already contain diagrams visualizing the interaction of several light and dark steps, we will not attempt to reproduce another one at this point. We will be commenting on various aspects of these schemes, and the reader is referred to one or more of them for clarification of the over-all relationships.

The necessity for cooperation of two "photosystems" in green plant photosynthesis has been established clearly from studies of the dependency of photosynthetic rates, and of fluorescence yield, on illumination by different wavelengths of light and combinations or alternations of these wavelengths. These "photosystems" may, according to one view, utilize their own specific pigment complexes for light absorption, and activate different parts of the photosynthetic electron transport chain. System I (terminology of Duysens*

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* Names refer to first authors of articles in this volume.

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and used by most participants; also "long wavelength system") is sensitized mainly by chlorophyll a; system II involves the accessory pigments plus some in vivo forms of chlorophyll a. The areas of greatest activity now cover the precise nature of the biochemical events set into motion by illumination of each of these pigment systems, and the mechanism for cooperation between the two.

Relatively little discussion or new information was presented at this meeting concerning the mode of energy transfer from the initial light absorbing pigment molecules, to the "quantum conversion centers" (alternatively - active centers, sinks). Past arguments concerning the existence of electron or hole migration in the chloroplast lamellae appear to have subsided, and resonance transfer is looked to by most for the method of energy transfer to the trapping centers (Duysens, Rabinowitch). The photosynthetic unit, according to one report (Kok) may be smaller than has been accepted until now.

No evidence was presented directly related to the intimate mechanism of the conversion of excitation energy to oxidation reduction energy at the trapping center. However, a very general underlying assumption is that the primary result of this mechanism is the creation of a negative potential electron donor and a positive potential electron acceptor.

Several laboratories report the direct observation of small amounts of long wave absorbing pigments (Olson, Butler). These arouse great interest because they could serve as sinks for migrating excitation energy; and hence they should be at the heart of any conversion center. For the same reason, attention is being paid to pigments having fluorescence emission bands at long wavelengths. These were recently observed at very low temperatures, and have been studied intensively both in vivo and in solutions of chlorophyll (Brody). The same considerations add great interest to the findings that a minor fraction of chlorophyll, having an absorption band at longer wavelengths is the only chlorophyll present in distinct spatial orientation (R. Olson).

Since quanta absorbed by the two pigment systems have complementary functions, it is clear that there must be two distinct conversion centers (although it is still possible that one conversion center is actually the photo-activated form of the other--a mechanism proposed by Franck). Studies

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of spectroscopic changes as affected by additions of co-factors, electron donors or acceptors, and inhibitors, clearly associate photosystem II with the high potential side of electron transport--specifically, with the release of oxygen. Photosystem I, on the other hand, appears to function in inducing electron flow at the negative potential end, providing the reductant for ferredoxin, TPN, the viologens, etc.

The conversion center for photosystem I is now generally agreed to be the long wavelength chlorophyll "P700" (Kok), probably a part of the oriented chlorophyll complex (Olson). Upon excitation by light energy it donates an electron to an associated, unidentified acceptor, which must then be quite a strong reductant. [The remaining radical might be responsible for one of the light induced EPR signals (Beinert)]. P700 apparently transfers one electron per mole, behaving as a redox reagent with a potential (+ 0.43 v) slightly higher than that of cytochrome f (+ .36 v) with which it appears to be associated in a charge transfer complex. This reaction is observed even at liquid nitrogen temperature (Witt, Chance). A third component, the recently discovered copper enzyme plastocyanin (Katoh) was reported to be another part of this trapping center complex.

Present knowledge or even speculations on the trapping center of photosystem II are meager by comparison. Studies of fluorescence yield (Duysens, Butler, Kok) clearly indicate a chlorophyll as being the final light collector but no unequivocal evidence has been found for a color change of this trap upon excitation.

It is usually assumed that photoact II creates an oxidant with a potential higher than + 0.8 volts, so that the oxidant by itself can oxidize water. However, there is no absolute need for such an assumption. An alternative presented at the meeting (Hoch), could involve the electron in a jump over any potential span, just so long as enough energy is provided to bridge the gap of + 0.4 volts between P700 and water.

The reductant made by photoact II according to most (but not all) is likely to have a potential of about 0.0 volts. More than one laboratory identifies this compound with plastoquinone (Witt, Trebst). However, the evidence, still indirect, is based mainly upon the assumption that absorption changes at 520 mu are due to an assumed complex between plastoquinone and another (carotenoid?) pigment. Some support

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is extended by experiments in which extraction of lipid components, including plastoquinone by hexane, leads to inactivation of both O_2 evolution and the 520 change.

Although the original paper of Hill and Bendall suggest cytochrome b_6 as the primary reductant of Photosystem II, the evidence so far has been discouraging (W. Bonner). Only under abnormal conditions is a light induced reduction of this carrier now clearly seen (J. Olson).

In complete photosynthesis the reductant of the second photoact reduces the oxidant of the first. The 0.4 volt potential gap between these two is believed to be bridged by exergonic electron flow, possibly coupled to ATP formation. Indophenol and other quinoid dyes, reduced by ascorbic acid, are able to donate electrons to some member of this region of the chain between the two photoacts. In that case electron flow to TPN or etc. is driven by photosystem I alone. Another locus of entry for electron donors other than water appears to be at the high potential end of the chain (Trebst, Witt). Such oxidations require both photoacts. A new approach in these investigations (Levine, Bishop) is the use of biochemical mutants with lesions in the electron transporting enzymes, combined with the application of specific electron donors, oxidants, or different wavelengths of light. On the whole these studies support the picture developed by other means.

Electron transport over the chain now partially defined, leads to phosphorylation of ADP. Net flow depends, of course, on addition of some soluble redox component, to which recently "phosphodoxin" (Black) a natural low molecular weight compound, can be added. The question why indophenol dyes were photoreduced without concomitant phosphorylation seems to be satisfactorily answered by the uncoupling effect of the oxidized dye (Keister, Avron).

Experience with phosphorylation accompanying the Hill reaction continues to show a P/2e ratio of 1.0 as a maximal limit. In view of the higher ratio demanded by the Calvin scheme for CO_2 fixation, and the demands in the cell for ATP to be used in growth and other synthetic processes, it would seem that in vivo phosphorylation should result in part from cyclic or pseudo-cyclic electron flow as well. Further evidence for photophosphorylation in whole algae, independent of oxygen evolution, came at this meeting from studies of acetate metabolism (Gaffron). Also, the photoinhibition of respiration observed with the mass spectrometer can be ex-

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plained by cyclic photophosphorylation maintaining a high ATP/ADP ratio inside the cell, thereby stopping electron flow in tightly coupled mitochondria (Hoch). This control of respiration by a photosynthetic process is thus analogous to the control over glycolysis by oxidative phosphorylation.

Cyclic electron flow with isolated chloroplasts has been observed with a physiological mediator, ferredoxin (Arnon). Its significance for the in vivo mechanism is obscure, because of the low rates, sensitivity to oxygen, and unique inhibition by low concentrations of antimycin A.

The actual mechanism of ATP formation is still mysterious. It was shown that a large pool of a non-phosphorylated, high energy intermediate can be formed at pH 6 in the light, and that the intermediate can be used for ATP formation subsequently, in the dark. The obligate role of this intermediate in ATP formation, or possible alternative functions for it, are still unknown (Jagendorf). Other aspects of the phosphorylation mechanism are exposed in the discovery of light induced ATPases in chloroplasts, and in the finding that the existence of high energy intermediates in the chloroplasts causes an increase in light scattering, probably due to structural changes (Packer).

With respect to the site of the phosphorylation mechanism in the electron transport chain, two possibilities are actively considered. One (probably the favorite for most) is in electron transfer from the primary reductant (E'_o of 0.0 V) made by photosystem II, to the P700 complex (E'_o of + 0.43 V). Experimental proof does not yet rule out the alternative possibility of phosphorylation associated with the re-oxidation of the low potential primary reductant formed by photosystem I which would have to have a potential of -0.6 V to obtain sufficient energy in re-oxidation by ferredoxin or by methyl viologen.

Since clear evidence as to the number or position of phosphorylation sites is lacking, care should be taken to avoid making definite commitments at the present time. Studies with inhibitors or uncoupling agents such as HQNO or CCP (Avron) as well as previous work with PCMB, do suggest that there might be more than one phosphorylation site.

The two photoreactions thus appear to be linked together by redox reactions and associated phosphorylation. An addi-

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tional question is how does each photosystem obtain its appropriate share of the absorbed quanta. Of two possible mechanisms the first one, which is supported by much of the data, assumes that the respective pigment systems and conversion centers are independent of each other. The second assumes that energy can "spill over" from system II to system I, but not vice-versa (Myers, Govindjee, Bannister and Hommersand). In the extreme case, this mechanism could operate with a single assembly of absorbing pigments if only given a switching device at the end point of energy transfer. One thing is certain--deciding about these possibilities is made much more difficult by the fact that it is never possible to irradiate system II by itself.

Further evidence as to the nature of the cooperation between the two photosystems comes from sensitive measurements of oxygen evolution (French, Fork, de Kouchkovsky; Whittingham). These experiments show that oxygen evolution due to irradiation of photosystem II is not initiated unless the chloroplasts or algae have been previously 'primed' either quickly by exposure to light activating system I or much more slowly by a dark incubation. Thus system I, according to this evidence, produces a precursor for system II to work on. Data of Govindjee and Whittingham, indicating that one sensitization band can operate in either photosystem, seem unexplainable as yet.

The biochemical details of the oxygen evolution process remain an area of almost complete ignorance. Earlier work showed that Mn is involved in the oxygen evolution system. Recent studies (Gaffron) suggest that flavin enzymes might be playing a part here, primarily from the discovery that the oxygen evolution inhibitor DCMU interacts very strongly with model photoreactions of soluble flavins. Some of the experiments of Trebst suggest that photo-oxidation of some of the quinones, or of ascorbic acid, may proceed by a reversal of the oxygen evolution pathway; however, all alternative explanations have probably not yet been ruled out.

The pathway of carbon continues to be a fruitful field of inquiry for a few groups. Increasing doubt seems to have developed as to whether the Calvin cycle is true in every respect in vivo. Most essentially, recent critical experiments seem to suggest that phosphoglyceric acid is not the only first product in photosynthesis; it appears that fixation of CO_2 onto ribulose diphosphate may, in part, produce

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a hexose molecule prior to the production of PGA. If so, this in turn reopens the question as to whether TPNH is the reductant in this phase of carbon fixation. The data of Krogmann indicate at best one TPN or two DPN molecules per photosynthetic unit in chloroplasts made by non-aqueous procedures, and this represents almost all of the TPN and half the DPN of the leaf. These figures suggest either that pyridine nucleotides are fully engaged in carbon fixation, or that they are simply bypassed. The question will not be fully answered, however, until the details of the initial carboxylation and reduction steps are elucidated (Bassham, Gibbs). At the present time, it appears highly suggestive that some sulfhydryl inhibitors (lipoic acid, arsenite) affect carbon fixation in the whole system although they are not known to inhibit any of the enzymatic steps established for the Calvin cycle in vitro.

A new direction in carbon metabolism has been clearly established for the two carbon pathway, starting from phosphoglycolic acid (presumably arising from pentose phosphate) (Tolbert, Whittingham). Under the usual natural conditions--high light intensities, 20% or more oxygen, low CO_2 concentrations--50% or more of the carbon flowing through photosynthesis can be shown to be running through this previously little explored two carbon pathway. A major exception to the generally accepted views on the pathway of carbon and indeed most other areas of photosynthesis, remains in the closely woven concepts of Warburg, lucidly described by Vennesland.

While this survey has so far considered analyses of pathways, further advances depend entirely upon a more complete knowledge of the participating constituents. For instance, plastoquinone A, implicated in electron transport by experiments involving extraction with hexane and re-addition, already appears to have two components active at two sites (Trebst). The occurrence of as many as eight structurally different quinones, most of them showing activity in reconstitution experiments suggests a more complex set of pathways than so far envisioned (Dilley). Work is proceeding on isolated cytochromes (Forti, Perini), and iron has been shown to be the electron carrier of ferredoxin (Fry). Properties and possible functions of plastocyanin are under investigation (Katoh). The nature of electron transport in the chloroplast surely depends on the existence of both aqueous and non-aqueous phases; hence, the fundamental importance of knowing the nature and function of the abundant

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lipid constituents (Benson).

Sophisticated methods of electron microscopy and small angle x-ray diffraction have been applied to a study of lamellar structure which must be responsible for the intricate transfer of quanta and charges to and within conversion centers (Menke).

In the long run the intimate details of the transformation of light energy must yield to an attack in which chloroplasts are broken down into their constituent parts and then reconstituted. The beginnings of this approach are seen in the reports of chlorophyll-protein complexes (Takamiya) particulate sub-units of the chloroplasts (Allen, Kahn), and even colloidal pigment suspensions (San Pietro, Vernon) retaining photochemical activity.

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