RESEARCH IN PHOTOSYNTHESIS

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RESEARCH IN PHOTOSYNTHESIS

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PREFACE

A meeting was held at Gatlinburg, Tennessee, October 25–29, 1955, to discuss some new observations and hypotheses in photosynthesis. The present volume owes its existence to the wish of the National Research Council, which sponsored the meeting, and of the National Science Foundation, which supported it financially, to have a record of the proceedings, permitting a greater number of scientists to benefit from the meeting than could be present at Gatlinburg.

A book of this kind, if it contained only the papers read at the meeting, would offer only the type of information usually supplied by the scientific journals, in which these papers probably would have been published; its only advantage would have been the collection of a number of related articles under one cover. The meeting was held, however, primarily to provide opportunity for extensive discussion; to convey its spirit, the editors decided to reprint more or less verbatim those parts of the discussion which suggested unsolved problems, or pointed out where different investigators disagree in the interpretation of recent observations. The material now contained in the book includes about one-third of the total discussion taken down by the stenotypist. This material has been edited from the point of view of grammar and clarity; for the rest, the remarks have been left unchanged, except where it is expressly stated that the text has been altered or new material added in proof. The reader will find that occasionally, the discussion following a paper returns to subjects already dealt with in connection with earlier papers. Nearly always it is obvious to what problem the questions and answers refer, and we did not try to transpose them. Transposition of discussion remarks has been made, however, in a few cases, where a whole paper was transferred in the book, into a group other than that in which it had been presented at the meeting.

Experience gained at an earlier meeting had taught us that discussions of this kind cannot be profitably extended beyond a period of about five days, and that, on the other hand, this period is not sufficient to cover thoroughly all facets of the problem of photosynthesis. The Subcommittee on Photobiology of the National Research Council decided therefore to omit from the agenda, among others, two big

topics in photosynthesis: the chemistry of the reduction of carbon dioxide, and the question of the smallest number of quanta capable of accomplishing this reduction. At the time of the meeting, the first of these topics had been treated in several reviews, which suggested general agreement concerning the experimental results and their interpretation. The second topic, too, was in a static condition —that of a disagreement which had persisted for twenty years.

The program was organized mainly around the problem of the primary photochemical process in photosynthesis——about which we know yet next to nothing—with the inclusion of those of the secondary reactions which seemed most likely to furnish clues to the mechanism of the primary process.

The editors wish to acknowledge the help of Dr. John Brugger in editing and transposing the discussion. Together with Miss Dolores Heffernan and Miss Marlene Roeder, he also took care of a great part of proofreading and indexing. It is a pleasant duty to express sincere thanks to all of them.

June 1957 H. G.

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Part I

ABSORPTION, FLUORESCENCE, LUMINESCENCE, AND PHOTOCHEMISTRY OF PIGMENTS *IN VITRO*

The Photochemistry of Chlorophyll *in Vitro*

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It is the primary act, the capture of light energy for chemical purposes, which makes photosynthesis the most important and the most fascinating of biological processes. Even if every enzyme, with its attendant substrate, product, and coenzyme, which enters into the biochemical cycles of the living plant were known, we might still be completely ignorant of the primary process which distinguishes photosynthesis from other biological processes. There is no known straightforward way by which the nature of the primary act can be determined. In fact, there are very few photochemical reactions of complex molecules in solution whose primary act has been unequivocally determined. In view of the difficulty of the problem, it appears worth while to investigate many different photochemical and spectroscopic properties of chlorophyll in solution, in synthetic and natural aggregates of chlorophyll molecules, and in the intact cell.

The study of dilute solutions of chlorophyll is certainly insufficient to establish the nature of the primary act of photosynthesis. However, knowledge gained from such study should enable the student of photosynthesis to reject many otherwise plausible interpretations of the primary act. This discussion is limited to recent developments of the photochemistry of chlorophyll solutions. Irreversible photochemical reactions of chlorophyll and reactions photosensitized by chlorophyll have been excluded. Although the irreversible reactions are of intrinsic interest to organic chemistry, they have no direct relation to normal photosynthesis. An adequate discussion of photosensitized reactions would require more space than is warranted by their importance. Furthermore, such reactions, occurring in dilute homogeneous solutions, cannot serve as reasonable models of photosynthesis.

ISOMERS, TAUTOMERS, AND ADDITION COMPOUNDS OF CHLOROPHYLL

Freed and Sancier (1) have shown that a tautomeric form of chlorophyll is stable at low temperatures, and they have studied, at intermediate temperatures, the tautomeric equilibria. This tautomerism is fast even at 150°K. The absorption maxima of the low-temperature forms are displaced to the red by about 300 A. The temperature at which the low- and high-temperature forms are present in comparable amounts is a sensitive function of the isomeric form of the chlorophyll. For example, the spectra of chlorophylls *b* and *b'* are almost identical at room temperature. Reducing the temperature to 75°K. changes both alike, so that the spectra of their low-temperature forms are also identical. However, the temperatures at which the tautomers coexist in equal amounts are 180° and 230°K., respectively, for chlorophylls *b* and *b'*. (More recent work by the same authors (*J. Am. Chem. Soc.*, *76*, 198, 6006 (1954)) indicates that the species that are stable at low temperatures are probably solvates rather than tautomers of the unsolvated molecules.)

Chlorophylls *a* and *b* form addition compounds with water, alcohols, amines, and other "bases." Similar adducts are formed by bases with metal-complexed porphyrins and chlorins, but not with pheophytins or metal-free porphyrins (2). These facts support Krasnovskii's contention (3) that the point of addition is the metal atom of the pigment. Unlike the other pigments, the fluorescent yield of chlorophyll is strongly affected by the addition of the base. Solutions of chlorophyll in pure dry hydrocarbons, or similar solvents, are practically nonfluorescent. The stability constants of compounds of chlorophylls, porphyrins, and chlorins with a given base do not differ by a factor of more than seven. As measured by these stability constants the oxygen bases are abnormally strong relative to nitrogen bases (4).

At −80°C. a solution of chlorophyll in isopropylamine is reddish brown, but when the solution is warmed to ordinary temperatures it becomes green and exhibits its normal spectrum (5). Except for a slow side reaction at the higher temperatures, the process is strictly reversible. The absorption spectrum of the low-temperature solution is identical with that of the Molisch phase test intermediate (6). This spectrum is similar to, but not the same as, the spectrum of chlorophyll in its triplet state, as formed by flash illumination (7). Since there is good reason for the belief that the phase test intermediate

is an ion, formed by the loss of a proton from C_{10} it appears plausible that the stable electronic state of this ion is a triplet.

PROPERTIES OF CHLOROPHYLL IN ITS SINGLET EXCITED STATES

Chlorophyll, excited by the absorption of blue light, emits only the usual red fluorescence. The molecule goes from its second to its first excited singlet state by a radiationless process of high probability (internal conversion). Since visible fluorescence having a yield as high as 10^{-3} is readily detectable and no blue or green fluorescence has been observed, the quantum yield, of this fluorescence is less than 10^{-3}. The actual mean life, $\tau = \varphi\tau^\circ$, where τ° is the intrinsic mean life, must be less than $10^{-3} \times 10^{-8} = 10^{-11}$ second. It is, therefore, most improbable that the second excited singlet state plays any direct role in the photochemistry of chlorophyll. In other words, the photochemical action of visible light should be independent of wavelength whenever chlorophyll is the only light-absorbing entity present. This does not necessarily apply to the absorption of ultraviolet light. Higher excited states may possibly enter directly into photochemical reactions, but such processes would not be related to normal photosynthesis.

The intrinsic mean life, τ°, of the first excited state of chlorophyll may be calculated (4) from its integrated extinction coefficient for "red" light. The actual mean life, τ, may be obtained (4) from measurements of the degree of polarization of fluorescent light in viscous solvents, by the use of Perrin's equation. Since the maximum quantum yields of fluorescence, φ°, are known (8) for chlorophylls *a* and *b*, the intrinsic life may be obtained from the actual life, as $\tau^\circ = \tau/\varphi^\circ$. The presently available values are listed in Table I. Weil's value for τ° (*b*) is based upon measurements made with benzyl alcohol as the solvent, assuming that $\varphi^\circ = 0.10$. Disregarding the values which Rabinowitch calculated from the early data of Prins, the values of the intrinsic mean lives of chlorophylls *a* and *b* fall in the ranges 3 to 1.3×10^{-8} and 4 to 1.6×10^{-8} second, respectively. The maximum quantum yield, φ°, for the fluorescence of chlorophyll *a* is 0.24, and it is independent of the solvent (8). The yield for chlorophyll *b* varies with the solvent, being 0.11 for ether and 0.06 for methanol. The corresponding values for the actual mean lives are $\tau^\circ(a) \cong 5 \times 10^{-9}$ and $\tau^\circ(b) \cong 3 \times 10^{-9}$ second.

TABLE I. Values for τ° of Chlorophylls *a* and *b*

Authors	Reference	Method	τ° (*a*) (sec.)	τ° (*b*) (sec.)
Rabinowitch	vol. 2*a*	Extinction values (Prins' data)	8×10^{-8}	9×10^{-8}
Forster	4	Extinction values (Zscheiles' data)	2×10^{-8}	4×10^{-8}
Jacobs	[a]	Extinction values	1.3×10^{-8}	1.6×10^{-8}
Brody	[a]	Extinction values	1.5×10^{-8}	2.3×10^{-8}
Weil	4	Polarization measurements	3×10^{-8}	4×10^{-8}
Stupp and Kuhn	9	Polarization measurements	1.6×10^{-8}	...

[a] These new values were presented by Dr. Rabinowitch at the Gatlinburg meeting (1955).

It is well established (4,10,11) that energy of electronic excitation can be readily exchanged between chlorophyll molecules, separated by distances as great as 50 A. This process, which is called induced resonance, leads to self quenching and concentration depolarization when it occurs between like molecules, and to fluorescence quenching and sensitized fluorescence when it occurs between unlike molecules. For chlorophyll solutions, self quenching is a quadratic function of concentration (4,10), $\varphi_{\max.}/\varphi = 1 + km^2$. It becomes appreciable at $m = 2 \times 10^{-3}$ and reaches its half value ($\varphi_{\max.}/\varphi = 2$) at about 1.5×10^{-2} *m*. It is not a function of the viscosity of the solvent, being practically identical in benzyl alcohol (4), acetone (10), and ethyl ether (10). Concentration depolarization follows a first-order law (4), $p^\circ/p = 1 + k'm$. The relative polarization, p/p°, equals one half at a concentration of 6×10^{-3} *m*. By a slight extrapolation of these data one would predict that φ and p should equal 0.5% and 1%, respectively, in a 0.1 *m* randomly oriented solution of chlorophyll in a highly viscous solvent. Since the concentration of chlorophyll is approximately 0.1 *m* in the grana it is interesting to compare these calculated values to the corresponding quantities which have been observed for chloroplasts and algae; 3% (Latimer) and 2% (Arnold), respectively. This near agreement suggests that the chlorophyll molecules are randomly oriented (at least in two dimensions), which is surprising in view of the evidence indicating that the pigment molecules in the grana exist in some ordered orientation.

In solutions which contain chlorophyll *a* and *b*, the fluorescence of *b* is quenched by *a* (10) and the fluorescence of *a* is sensitized by *b*

(10,11). The probability of the transfer of excitation from *b* to *a* leading to the fluorescence of *a* reaches a maximum of 0.5 at about 10^{-3} *m*, when the concentrations of *a* and *b* are equal (10,11). Concentration quenching becomes dominant at higher concentration and reduces the probability of sensitized fluorescence to a negligible value at 10^{-2} *m*. Sensitized fluorescence is a well-known phenomenon in the intact plant.

The fluorescence of chlorophyll solutions is strongly quenched by oxidizing agents (such as quinones, aryl nitro compounds, nitroso compounds, and oxygen) but is not detectably quenched by a wide variety of reducing agents (including thiourea, urethane, hydroquinone, ascorbic acid, etc.). Quenching by the oxidizing agents is chiefly a diffusional process. The polarization of fluorescence increases with the degree of quenching in accord with Perrin's equation (4). The rate constants corresponding to the bimolecular quenching reaction can be calculated from the ratio of the Stern-Volmer quenching constant, k_Q, to the actual mean life of the excited state, τ. If the rate of the reaction is determined by diffusion, k_Q/τ should be approximately equal to $k_D \cong 8RT/30\eta$, where η is the viscosity of the solvent expressed in centipoise. A few such values for chlorophyll *a* at 25° C. are listed in Table II. In these calculations a value of 5×10^{-9} second has been assumed for τ. The agreement is fairly good, but there is probably some static quenching, which is more apparent in viscous solvents (4). The data are not sufficiently extensive to justify a more extensive analysis.

TABLE II. Bimolecular Quenching Constants

Quencher	Solvent	k_Q	k_Q/τ	k_D
O_2	Ethanol	35	7×10^9	6×10^9
$C_6H_4O_2$	Methanol	120	2.4×10^{10}	1.2×10^{10}
TNT	Methanol	100	2×10^{10}	1.2×10^{10}

PROPERTIES OF CHLOROPHYLL IN ITS TRIPLET STATE

Kinetics studies of photochemical reactions sensitized by chlorophyll demonstrate that it is usually not the fluorescent (i.e., first excited singlet) state but rather a metastable state of chlorophyll which is directly responsible for the photochemical action. When the reactant is present at high concentration in a fluid medium or exists

as a thermally stable complex with the chlorophyll, the fluorescent state of the chlorophyll molecule can play a direct part in the photochemical reaction. Such reactions are, of course, accompanied by marked quenching of the fluorescence.

By analogy with fluorescein and with aryl hydrocarbons, it is commonly assumed that the metastable state is the lowest triplet state of the molecule. Most unsaturated compounds exhibit phosphorescence when they are dissolved in glassy media. Recently Becker and Kasha (12) have confirmed the existence of a weak, near infrared phosphorescence of chlorophyll *b*. The present status of the subject may be summarized as follows. No temperature-dependent phosphorescence (with the same wavelength distribution as fluorescence) has been observed for either chlorophyll under any conditions. No phosphorescence of any type has been observed for chlorophyll *a*. Chlorophyll *b* shows a temperature-independent phosphorescence (i.e., long-lived fluorescence) at 8650 A, which has a half-time of about 3×10^{-2} second and a quantum yield less than 10^{-3}. The observed wavelength corresponds to an energy difference between the ground and the metastable states of 33.0 kcal.

Organic molecules are phosphorescent only when they are dissolved in rigid media or adsorbed on solids. Therefore, the measurement of phosphorescence is not well adapted to the investigation of the chemistry of the triplet states of such molecules. This is especially true for chlorophyll, whose phosphorescence is very weak. The flash-photolytic method, which was brought to its present state of development by Dr. George Porter (13) at Cambridge, is a much more effective tool for such studies.

Preliminary studies (14), using a flash of actinic light and a steady monochromatic beam for analysis, were made by Dr. V. Ryan. He succeeded in demonstrating that a large fraction of the chlorophyll in a dilute solution is raised to its triplet state by a single intense flash. Under his experimental conditions the half-life of this state was about 3×10^{-4} second. Dr. Linschitz (15) who used an improved apparatus of this type, confirmed in general Dr. Ryan's findings. He also obtained the absorption spectrum of chlorophyll *a* in its triplet state, and showed that it has a maximum on the long-wavelength side of the red band of normal chlorophyll. Preliminary absorption spectra of the triplet states of the chlorophylls and related compounds were obtained by Livingston (7) using the

flash-photolytic, flash-spectrographic apparatus of Porter and Windsor. Figure 1 is a plot of the visible absorption spectrum of chlorophyll *b* in pyridine, which was obtained by Mr. Fujimori at the University of Minnesota. He has also confirmed Linschitz' result that the triplet state has a broad absorption maximum in the near infrared; about 7500 A for chlorophyll *b* in benzene.

It has been reasonably well established that the disappearance of the triplet state occurs, at least in part, by a bimolecular, self-

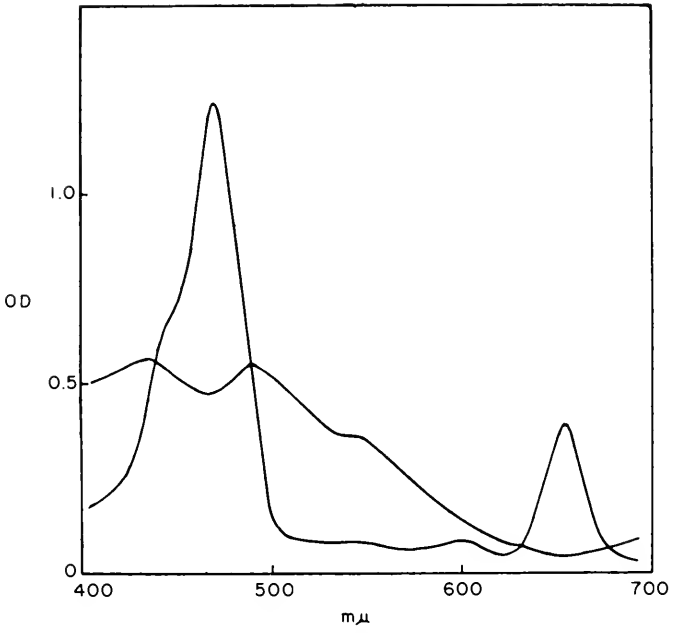

Fig. 1. Absorption spectra of the singlet and triplet states of chlorophyll in pyridine (1.3×10^{-6} *m*).

quenching process in fluid solvents. The study of the reactions of the triplet state has scarcely begun. It is known that molecular oxygen quenches the triplet state efficiently; presumably, at every encounter. Allylthiourea has little if any effect upon the mean life of the triplet state. Further investigation of these reactions should be of great value in the interpretation of the primary act of photochemical reactions.

REVERSIBLE PHOTOCHEMICAL REACTIONS OF CHLOROPHYLL

Anaerobic solutions of chlorophyll in methanol, ether, etc., but not in hydrocarbons show rapid reversible changes in their absorption spectra when they are strongly illuminated. Under experimentally realizable conditions, the maximum change in the absorption of monochromatic light is less than 1%. Since the percentage change is proportional to the square root of the intensity of the absorbed light, we may conclude that the labile product is a pair of radicals or ions which reform chlorophyll by recombining. The absorption spectrum of this intermediate has been determined (14) only very crudely, but appears to resemble that of the triplet state (7). Although this reaction is intrinsically interesting and may be related to the primary act of photosensitized reactions, the last publication dealing with the subject appeared in 1950 (16).

It was observed by Linschitz (15) that chlorophyll, in a rigid solvent (EPA at liquid nitrogen temperatures) containing a quinone, is photochemically transformed into a product which is stable at low temperatures but reverts immediately to normal chlorophyll when the glass is allowed to warm to its softening point. The absorption spectrum of this product is, likewise, generally similar to that of the triplet state.

The earlier finding of Krasnovskii that chlorophyll can be reversibly, photochemically reduced by ascorbic acid, when dissolved in pyridine, has been confirmed by A. S. Holt and E. Rabinowitch and by Evstigneev and Gavrilova (17b). The latter authors also observed this reaction in toluene with phenylhydrazine as the reducing agent. They have further shown that two labile intermediates are formed, and have postulated that the maximum at 585 mμ is due to a neutral molecule or radical which ionizes in basic solution with the resulting appearance of a maximum at 518 mμ. They measured the fluorescence and low-temperature phosphorescence of these substances.

Discussion

Rabinowitch: How does the absorption curve of the triplet state compare with that of ionized chlorophyll?

Livingston: The absorption spectra of the ions, of the triplet state, of Krasnovskii's reduced form, and of Linschitz' chlorophyll-quinone intermediate, are similar but not identical. They show distinct differences; but in each case the two singlet peaks disappear, the Soret band is replaced by what looks like two

separate peaks, and there is apparently a new absorption band in the far red or near infrared.

Rabinowitch: There seems to be a definite distinction in that metastable (triplet) chlorophyll *a* has a large band at 475 mμ and only a slight indication of a band at 525 mμ (or somewhere in that region), while Krasnovskii's reduced chlorophyll has a very prominent peak at 515 mμ, but at 475 mμ it shows only a negative effect (decrease in absorption). The ionized (and also perhaps the oxidized) chlorophyll *a*, as well as the metastable and the reduced form all have a band at 515–525 mμ, but I think there is a definite distinction between the metastable and the reduced form at 475 mμ. This, I think, is important, particularly in connection with what we will report later on the changes in the absorption spectrum of illuminated *Chlorella* cells.

Duysens: Mr. Goedheer has made some careful measurements of the polarization spectra of the fluorescence of the chlorophylls, and his interpretation of these measurements is that the Soret band consists of two different bands with transitions perpendicular to each other. There is between the Soret band and the red absorption band of chlorophyll and of bacteriochlorophyll another weaker electronic band, with a transition perpendicular to that of the red absorption band.

Rabinowitch: Kuhn has assigned one of the weak chlorophyll bands in the green to a different electronic transition. This could not be confirmed at Utrecht. On the other hand, another band in the yellow region appeared to belong to a separate electronic transition. We, too, could not confirm Kuhn's result, and thought that it may have been due to contamination of his chlorophyll with pheophytin, which has a stronger band in the green than chlorophyll.

Linschitz: We have a flash spectrum which shows a peak for the metastable state somewhat further toward the green, at about 530 mμ.

Rabinowitch: Do you have a prominent peak at 475?

Linschitz: We have a suggestion of a shoulder at 475, but we have a higher peak further out toward the green, where Livingston found a somewhat lower absorption.

(*Note added in proof:* Since this work was prepared for press, Dr. Linschitz completed a series of careful measurements of the absorption spectra of the chlorophylls *a* and *b* in their triplet state. Chlorophyll *a* in its transient state has its main peak at 485 mμ and a lesser peak at 385 mμ. The absorption extends throughout the visible, being still appreciable at 750 mμ. There does not appear to be a maximum near 530 mμ, nor on the long wavelength side of the normal red peak.—R. L.)

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The Electronic Spectra of Chlorophylls and Related Molecules

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The present discussion will deal only with recent advances in the knowledge of the absorption and emission spectra of the chlorophylls and their derivatives. The nature of the triplet state, the general significance of quantum yields, the effect of electric and magnetic fields, and the like have been discussed elsewhere.

ABSORPTION SPECTRA AND DEDUCTIONS

The interpretation of the fluorescence activation of the chlorophylls by Livingston, Watson, and McArdle (3) has been previously discussed (1). However, there are several additional factors to be pointed out.

Freed and Sancier (5) have shown, from absorption data, the presence of various solvates and temperature-dependent isomers of chlorophylls and related compounds. From these data, it was determined that if solvation occurs at the magnesium atom of chlorophylls, it also occurs at or near the two corresponding hydrogen atoms of pheophytin. Russian workers (6) emphasized that the difference in activation of fluorescence between pheophytin and chlorophyll was due to the fact that hydration of magnesium was necessary. Thus, the fluorescence of pheophytin, which contains no magnesium, was insensitive to the presence or absence of water vapor. Although previously indicated, with consideration of the work of Freed, it now may be emphasized that the presence of the magnesium may well cause the perturbation of the electronic system of the chlorophyll molecule necessary for the facts concerning fluorescence to become observable. Consequently, the observed results concerning the effects of the presence or absence of water on the fluorescence may be only coincidental facts of interest accompanying the real cause of the

difference in the two molecules, that is, the effect of the presence or absence of magnesium in the molecules.

EMISSION SPECTRA AND DEDUCTIONS

It has been recently shown that the phosphorescence of chlorophyll *b*, first reported by Calvin and Dorough (4), is a *bona fide* emission occurring at 8650 A (2). An analog of chlorophyll, pheophorbide *a*, has a strong fluorescence and no phosphorescence. However, in the divalent copper complex of pheophorbide *a*, the fluorescence is completely quenched and only a strong phosphorescence is observed. Moreover, the phosphorescence occurs at 8675 A. The complete quenching of fluorescence in the metallophosphorbide and the like positions of the phosphorescent emissions indicate that the phosphorescences observed in the pheophorbide complex and in the chlorophyll are the lowest triplet to singlet emissions.

Although intrinsic phosphorescence was not unequivocally observed from chlorophyll *a*, there was indication of this type of emission. The difference between chlorophyll *a* and chlorophyll *b* is primarily due to the greater sensitivity of chlorophyll *a* to photodecomposition with accompanying spurious emissions. The difference in photosensitivity of chlorophylls *a* and *b* was further borne out by Dr. Linschitz in his report at the Conference on Photosynthesis in 1955. Chlorophyll *a* is being subjected to further investigation.

Although the quantum yield of phosphorescence was low (<0.1) for chlorophyll *b*, the extrapolation to the *in vivo* system allows important details to alter the value of the quantum yield. Earlier considerations by Becker and Kasha (1) indicated the very important possibilities of strong intermolecular spin orbital perturbations which may take place. These factors could substantially increase the quantum yield of phosphorescence.

The energy available in the lowest excited level corresponding to the wavelength of the phosphorescent emission is about 33 kcal. per mole. The energy available in the lowest singlet state is approximately 43 kcal. per mole. Although the energy available in the singlet state is higher, a consideration of the amount of absorbed energy reemitted from the singlet state deemphasizes this factor. Approximately 90% of the absorbed energy is unaccounted for in terms of reemission; 10% of the absorbed energy reappears as fluores-

cence (7). The now confirmed presence of phosphorescence accounts, in part, for the remaining energy and it may, in fact, account for all of it *in vivo*.

Certainly the advantageous properties of the triplet state compared to the singlet state such as longer lifetime warrant serious consideration and further investigations of the photosynthetic process with the triplet state in mind. Moreover, the energy availability is concurrent with the seeming requirements for subsequent chemical reactions in photosynthesis as offered by recent authors. Practically all work in the field of photosynthesis and photochemistry has been concerned with the minor part of the energy and its possible mode of utilization. It seems more reasonable to be concerned with the fate of 90% rather than with 10% or less of the available energy.

Discussion

Linschitz: The phosphorescence yield *in vitro* must be of the order of 70% or so. Could I ask if you are going to account for the chemistry *in vitro* by the triplet state? Regardless of what happens *in vivo? In vivo* do you think that the measurement could possibly bring the yield up that high?

Becker: I won't say yes and I won't say no.

Rabinowitch: You mentioned that the photochemical energy yield in photosynthesis is up to 70%. That does not mean that the yield of phosphorescence is 70% in the absence of photosynthesis, because the metastable state can be destroyed by internal conversion.

Linschitz: That is true, but if you put in copper you can bring up the phosphorescence to very high values.

Becker: The chances are the yield is not low. I am not saying there is a 100% quantum yield of phosphorescence. All I am saying is that the emission is 100% phosphorescence. In other words, we obtained no fluorescence.

Duysens: Is it possible that the quantum yield of phosphorescence is very small—say, 0.1 per cent?

Becker: No, it wouldn't be that low. If I had to give an estimate, I would certainly say not less than 50% yield, on the basis of slit widths, exposure times, and plate darkening.

Duysens: There is no actual basis except it is a sort of culmination of times of exposure, etc., giving some indication of intensity of emission.

Jacobs: I would like to put in a plug for the importance of the hydration of metal atoms for reactions *in vivo*. A lot of work has been done on oxygen exchange with water in the *in vivo* reactions of electron transport systems. It is one of the first changes to disappear when the phosphorylation activity associated with electron transport disappears. I think you underestimate it. This hydration of metal atoms may eventually cause loss of a phenomenon which will turn out to be important in photosynthesis as well as other processes associated with electron transport. This is only a suggestion.

Weigl: I would like to ask if anyone has tried measuring phosphorescence intensity *in vitro* during a chlorophyll photosensitized reaction. It seems to me that it is very important to demonstrate that it goes down very appreciably.

Becker: I haven't and I don't know of anyone who has.

Weigl: It seems to me that ought to be done.

Livingston: I would like to make a slightly different interpretation of Dr. Becker's own data. I think these data show that the yield of phosphorescence, meaning not the transfer to the triplet state but the actual yield of energy as phosphorescence, must be less than 0.001. I am taking this in part from a statement of Kasha's, that the observed intensity of the phosphorescence was less than the infrared contribution to the fluorescence. The infrared contribution of fluorescence is certainly less than a few tenths of a per cent: so if that statement is true, it would show that the phosphorescence has a yield of 0.001 or less. Also you stated that the fluorescence in this infrared region obscured the phosphorescence; so that the fluorescence intensity must be much greater than the phosphorescence, which again proves that the observable phosphorescence, the emission of phosphorescent radiation, has a quantum yield less than 0.001; nothing like 0.1.

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Fluorescence Spectra of Protochlorophyll, Chlorophylls *c* and *d*, and Their Pheophytins

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The fluorescence spectra of pheophytins *a* and *b* (1) as well as of pheophytin *d* in Fig. 1 are very similar to those of the chlorophylls from which they are derived except for a 2 to 13 mμ shift to longer wavelengths.

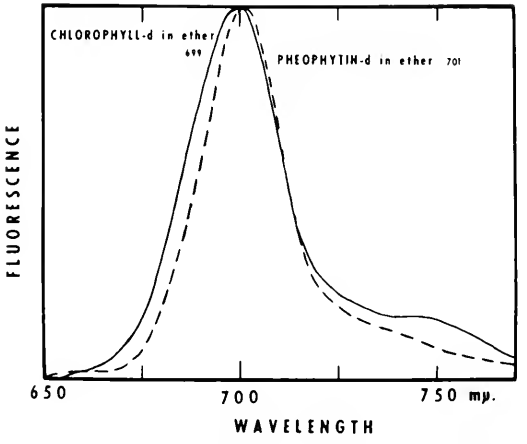

Fig. 1. The fluorescence spectra of chlorophyll *d* and pheophytin.

Acid-treated chlorophyll *c* and protochlorophyll, however, show larger shifts of the main fluorescence band, and a greatly increased height and corresponding shift of the second fluorescence band, as shown in Figs. 2 and 3. The absorption spectra of the same preparations (2) and some other chlorophyll fluorescence spectra have been published (3) or appeared elsewhere (1).

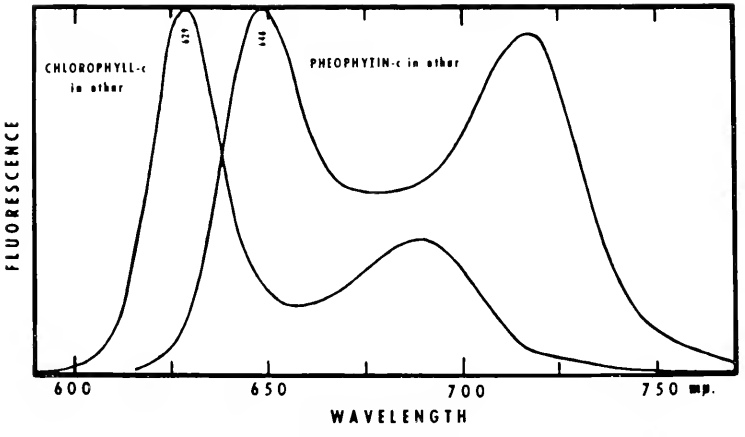

Fig. 2. The fluorescence spectra of chlorophyll *c* and pheophytin *c*.

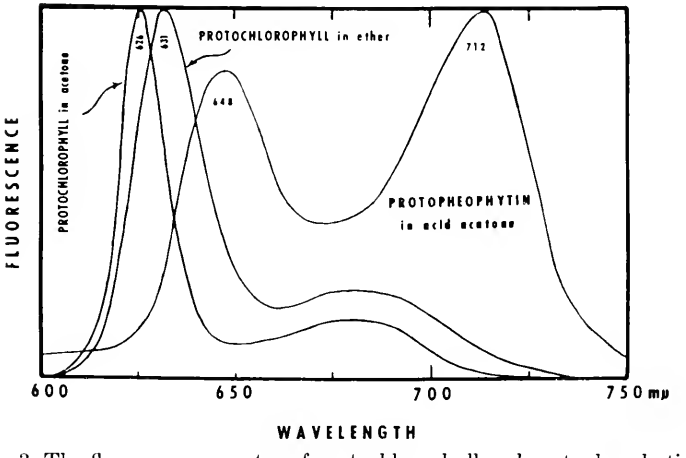

Fig. 3. The fluorescence spectra of protochlorophyll and protopheophytin.

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General Remarks on Chlorophyll-Sensitized Photochemical Reactions *in Vitro*

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The discussion of photochemical reactions sensitized by chlorophyll *in vitro* shows that the only reactions which proceed with high quantum yields are exothermic or slightly endothermic. More endothermic reactions have very small yields because these require that the bulk of the excitation energy of the chlorophyll be stored as chemical energy. In the process of sensitization, the excitation energy must be greater than the sum of the heat of activation and the chemically stored energy. Keeping this in mind, one finds it astonishing that the latter type of reaction takes place. However, the difficulty vanishes if one assumes that, for the first photochemical step, the energy of two quanta rather than of one quantum is utilized. The transfer of a hydrogen atom from the chlorophyll to a weak oxidant is an example. We suppose that the hydrogen to be transferred is the one bound to C_{10} in ring V of chlorophyll. The excitation energy of 41 kcal. (first excited singlet state) permits the transfer only to a strong (not to a weak) oxidant. When chlorophyll is in the lowest triplet state (corresponding to approximately 30 kcal.), even the transfer to oxidants as strong as molecular oxygen or quinone in one act becomes energetically impossible. (If one considers an electron transfer instead of an H-atom transfer, the energy situation becomes still more unfavorable.) There is a possibility that one hydrogen atom may be transferred in two chemical steps. However, the assumption which best fits the kinetic data for reactions *in vivo* is that two quanta are utilized to excite *one* chlorophyll molecule into an excited triplet state, whereby between 60 and 70 kcal. become available for *one* photochemical act—in our case the transfer of one hydrogen atom.

That can be achieved by the following steps: (*1*) Excitation of a chlorophyll molecule by light absorption into the first excited singlet state. (*2*) Internal transition into the lowest metastable, long-lived triplet state. (*3*) Excitation of the chlorophyll in its metastable triplet

state to the next higher triplet state by sensitized fluorescence. The energy needed for this process comes from a neighboring chlorophyll molecule in the first excited singlet state. (*4*) Utilization of the energy difference between the excited triplet and the singlet ground state for photochemistry.

I am not competent to discuss the theory of what Franck calls sensitized fluorescence, but let me at least outline the experimental evidence for its existence and the usual criteria for a probable transition of this sort.

When chlorophyll absorbs red light it is raised to its first excited (or fluorescent) state, usually with some extra vibrational energy. This excess vibrational energy is quickly lost, bringing the electronically excited molecule into thermal equilibrium with its surroundings. There is a large probability (between $^3/_4$ and 1) that the excited molecule will not emit a photon of fluorescence light but will be transferred by a process of internal conversion into its lowest triplet level. In this state, the life of the molecule will be long compared to its life in the fluorescent state. When two similar molecules are close together but not in actual contact, there is a certain probability that, if one is electronically excited, it can lose that energy and the energy of excitation will appear in the second molecule. This can also occur when the molecules are unlike, if certain requirements are satisfied. An empirical requirement is that there should be a strong overlap between the emission band of the first (primarily excited) molecule and the absorption band of the second molecule. The probability of such a transition caused by sensitized fluorescence is great when there is a large overlap between the emission band and the absorption band. For this reason, one might expect a greater probability of such radiationless transfer between unlike molecules than between like molecules. We find that there is a higher probability for energy transfer from chlorophyll *b* to *a* than for a transfer from *a* to *a* or *b* to *b* because the overlapping of the fluorescence band of *b* and the absorption band of *a* is great.

Because the visible absorption spectra of the triplet state and of the Molisch phase test intermediate of chlorophyll are similar, Franck predicted that the triplet state, like the phase-test intermediate, would have an appreciable absorption in the near infrared close to the red maximum of the singlet absorption. This prediction was verified by Linschitz and more recently by Fujimori. There is, therefore, a strong overlap between the fluorescence of singlet-state chloro-

phyll and the absorption of its lowest triplet state. This condition strongly favors a radiationless transition of the excitation energy from the first-excited singlet to the lowest triplet level of chlorophyll.

If I understand Franck's suggestion, it is that in grana, where the molecules are close together and where they are probably ordered, there is a very high probability of transfer of the energy of excitation from one molecule to another, that eventually this energy will end up in a sink and that sink can be a chlorophyll molecule already in its triplet state.

This doubly excited molecule should have a short life, comparable with that of the fluorescent state. It is, therefore, most improbable that the molecule in its excited triplet state could take part in an efficient chemical reaction which is controlled by diffusion. In other words, if this short-lived molecule is to use its energy efficiently in a chemical reaction, it must have come in contact with its reaction partner before it has received its second quantum of energy.

Even at the low dye concentrations usually chosen in experiments on photochemical processes *in vitro*, the probability of exciting the higher triplet state is not too small to allow the low rates of endothermal photochemical reactions. If this hypothesis is correct, the quantum efficiency of such processes should become much higher whenever chlorophyll is adsorbed on suitable surfaces in such a way that the average distance between the chlorophyll molecules is very small. This should raise the probability of Step 3 without interference from quenching impacts between the chlorophyll molecules.

Discussion

Weigl: There is a rather stringent restriction on the lifetime of this doubly excited state, unless it is a very unusual species (for instance, one stabilized by some prior chemical attachment). Internal conversion to the lowest member of any series of states of a given multiplicity is usually very fast—it proceeds in about 10^{-12} to 10^{-13} second. Therefore, a doubly excited triplet state would have to engage in chemical reaction within roughly 10^{-12} second, before the molecule drops to its lowest triplet state and the energy of the second quantum can be lost to molecular vibration and heat.

Rabinowitch (*remarks in proof*): If this were so, no molecules could have a fluorescence yield 0.01%.

Brugger (*remarks in proof*): Prof. Franck believes that the transition $tr^* \rightarrow tr$, first excited triplet to ground triplet state, resembles $s^* \rightarrow s$, first excited singlet to ground singlet state. In each case, the transition is to the ground state of a given multiplicity series. One expects the lifetimes of tr^* and s^*, as well as the fluorescence yields for $tr^* \rightarrow tr$ and $s^* \rightarrow s$, to be similar. In addition, tr^* can pass non-radiatively to s^* just as s^* passes to tr.

Lumry: In other words: the conditions should be as stringent for the triplet-triplet transition as they are for a singlet-singlet transition.

Brugger: Figure 1 may aid in clarifying some points.

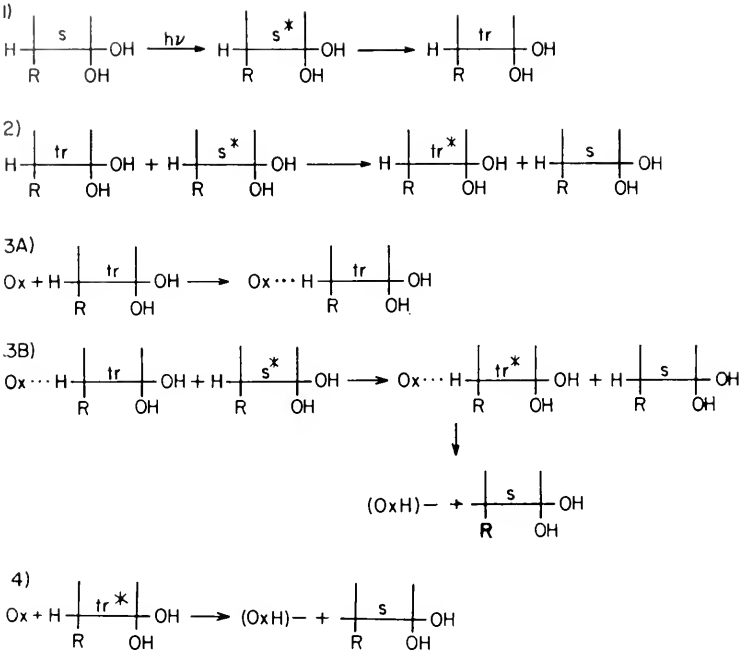
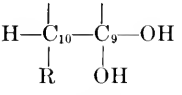


Fig. 1

The grouping



represents a hydrated form of chlorophyll and specifically shows hydration of the carbonyl group in ring V. In reaction sequence (1) of Fig. 1, chlorophyll is excited to the first excited singlet state s^*. Then, by a nonradiative transition, the singlet state crosses over into the ground triplet state *tr*. By a process of sensitized fluorescence, as shown in reaction sequence (2), this same molecule in its triplet state *tr* absorbs a quantum of energy liberated when some nearby chlorophyll molecule in the first excited singlet state s^* reverts to its ground singlet state *s*. This method of raising chlorophyll molecules to the excited triplet state tr^* is a unique feature of Dr. Franck's theory. The triplet state has an absorption far enough in the red to make this process feasible. Dr. Franck believes that adsorption (reaction (3A)) of some molecule, oxidant and/or enzyme, on the chlorophyll in the triplet state *tr* would make it an even better sink for light energy, viz., increase the overlap between singlet fluorescence emission and ground triplet absorption. As a second consequence of such adsorption, one could have the mole-

cule to be reduced already present. Thus, for photochemical reactions of chlorophyll *in vitro*, Dr. Franck postulates adsorption of the oxidant by chlorophyll in the metastable triplet state, as shown in equation (3A), followed by excitation of the chlorophyll complex to the excited triplet state *tr** by sensitized fluorescence and break up of the complex to form a reduced oxidant radical and a chlorophyll radical, as shown in reaction sequence (3B). The chlorophyll radical is presumed to lose an OH and rearrange to form a chlorophyll molecule. In photosynthesis *in vivo*, Dr. Franck expects that the relatively long-lived triplet state chlorophyll would adsorb (or become associated with) an oxidant, which would eventually be reduced by the H on C_{10}, and with an enzyme, possibly a cytochrome, which would carry off one of the OH's on C_9. This process would produce H-Ox- and HO-Enz- radicals and leave the chlorophyll molecule in the ground state.

One might expect that a chlorophyll molecule in the excited triplet state *tr** would transfer the H-atom directly to an oxidant during a collision, as shown in reaction (4). The state *tr** has sufficient energy to reduce quinone, ferricyanide, oxalate, PGA, DPN, etc., by photochemical H-atom transfer. For the usual concentrations of the molecules, however, the yield would be very low. One has the same problem with the excited triplet state *tr** as he has with any higher excited state, namely, if the excited molecule does not collide and react with another molecule within *ca.* 10^{-12} sec., the excitation energy will be dissipated as heat or light and the yield of the photochemical reaction will become small. Dr. Franck does not intend to endow the excited triplet state of chlorophyll *tr** with an especially long lifetime, which is what a good yield by reaction (4) would require. He postulates the reaction sequence (3A) and (3B) in which a reaction complex is formed by the long-lived metastable triplet state *tr*, thus not only obviating the need for a collision with the short-living excited molecule *tr** but even increasing the probability of exciting the chlorophyll to the *tr** state.

Duysens: It seems to me that if you illuminate with weak light, you have a low concentration of triplet; at higher light intensity you would have more triplet. So you would think, if the transfer takes place through induced resonance, that the efficiency of transfer from the excited singlet would be greater at higher light intensity than at a lower light intensity and that would mean the fluorescence yield of the chlorophyll would decrease at the high light intensity. Experiments indicate, however, that the fluorescence yield is essentially constant. I wonder how this difficulty can be resolved?

Brugger: At each light intensity, one should come to a steady state in which the ratio of molecules in the first excited state to those in the ground metastable state is constant. The fluorescence yield is also roughly constant. I expect that the rate of populating the excited metastable state should be proportional to the irradiation intensity. It would seem that the percentages of the molecules in the first excited singlet state which (*1*) fluoresce, (*2*) populate the ground metastable state, or (*3*) excite the metastable state by sensitized fluorescence should be roughly the same at all except the lowest light intensities. I do not feel that higher intensities would populate the metastable state disproportionately heavily nor that the efficiency of sensitized fluorescence would be much greater nor that fluorescence would be markedly quenched.

Rosenberg: May I rephrase Dr. Duysens' question to see whether I understand

what he is asking? I think he is saying that, if you populate the ground triplet state to a greater extent, which you presumably do by stronger irradiation, do you increase the drainage from the excited singlet.

Duysens: In which case you would lower the fluorescence yield.

Rosenberg: But I don't think that that happens to any appreciable extent. The steady-state population of the first fluorescent state and of the lowest triplet state would both be proportional to the light intensity in close approximation.

Duysens: I do not think that Dr. Rosenberg's remark is correct, because the drain is proportional not only to the number of excited singlets but also to that of non-excited triplets.

Rosenberg: But also, at the same time, you are increasing the total number of quanta absorbed per second so that the fractional yield of the molecules which are fluorescing may remain the same. It is the fractional yield of fluorescence that is constant experimentally.

Lumry: If all the processes out of the first excited state are of the first order in the concentration of the molecule in the excited state, then the only way you can change the amount of fluorescence is by changing the crossing point. Unless you are postulating a back reaction from the triplet into the singlet, the fraction of the fluorescence yield isn't going to change, no matter what you do to how many triplet molecules.

Duysens: If you have no triplet, there is no loss of fluorescence. Assume the fluorescent yield is, say, 50%. If you have a very efficient transfer, then the fluorescent yield is zero.

Lumry: This just does not have anything to say about the lifetime of the triplet or the population. It is a question of whether it can get out of the singlet into the triplet.

Duysens: But my contention is that the efficiency of transfer to the triplet state is proportional to its concentration. If it is two million, then you have twice as great a transfer as if you had one million. Then the fluorescence yield of chlorophyll changes. If the process of transfer is fairly efficient, it may change by, say, 50%.

Rosenberg: I think there is a self-balancing mechanism in Franck's picture which answers Duysen's question. In the Franck picture, the metastable state would be formed at a rate proportional to the concentration of the fluorescent state material. It would also disappear at a rate proportional to the concentration of the fluorescent state material. Therefore, the steady-state concentration should be independent of the light intensity and it should be simply a ratio of rate constants, i.e., the rate constant of formation divided by the rate constant of loss. So we don't have to worry about the variation of population of the lowest triplet state with the light intensity.

Arnold: This is true until the rate is so slow you have to worry about the lifetime of this triplet.

Rosenberg: Yes, I am forgetting the natural decay of the triplet.

Duysens: But this triplet state is supposed to react chemically.

Rosenberg: No, only in its excited state.

Duysens: So the concentration remains constant?

Rosenberg: Yes, as long as you don't worry about the natural decay of the lowest triplet state.

Rabinowitch (*expanded in proof*): As long as each *tr* has the chance of getting a second quantum by sensitized fluorescence (resonance transfer) during its lifetime, i.e., as long as *every* triplet excitation becomes a double excitation, the proportion of singlets which will succeed in emitting fluorescence will be independent of light intensity. The fluorescence yield will, however, begin to increase when the light intensity becomes so low that, during the lifetime of *tr*, no quantum will be absorbed by a singlet molecule near enough for the excitation energy to be snatched away by the *tr*. This intensity must depend on two things—the lifetime of *tr*, and the range over which *tr* can grab quanta by resonance transfer. If *tr* lives 10^{-3} sec. and can grab a second quantum from 10^3 singlet neighbors, the critical light intensity will be that at which each chlorophyll molecule absorbs a quantum once a second—which corresponds to a quantum flux of the order of 3×10^{15} photons near the peak of chlorophyll absorption, or to several thousand lux of white light. Experiments indicate no decrease of fluorescence yield with increasing intensity in this region. If the phenomenon exists, it must be over-compensated by an increase in yield due to other causes.

Weigl: The theory requires that at the very lowest light intensity you would get a very sharp dropping of the quantum yield of whatever reaction evolves.

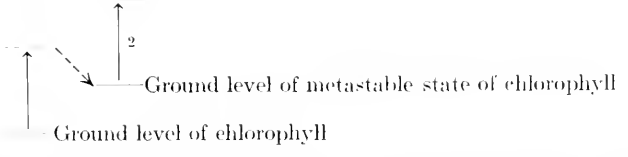
Gaffron: One should perhaps point out that Franck's theory recreates, in a sense, the concept of photosynthetic units. Each time this triplet state appears it becomes momentarily a sink and the nearby excited molecules can deliver their first singlet-state energy into it.

Strehler: Does anyone have evidence for the appearance of a band in illuminated chlorophyll solutions in the red region of sufficient intensity to absorb fluorescent light from the normal chlorophyll singlet-singlet transition?

Rabinowitch: Wasn't it reported today that the metastable state has an increased absorption in the near infrared?

Strehler: It was the solvation band that was out further.

Linschitz: The absorption spectrum of the metastable state of chlorophyll, as measured in our flash experiments, shows a band in the far red, just beyond the main red band of chlorophyll itself. This new absorption band corresponds to a transition from the ground level of the metastable molecule to what is probably the first excited level, as follows (transition 2).



The evidence indicating the existence of this band is completely unambiguous. (See Fig. 2*B* in our paper, for example.)

Weigl: It is in the position required by Franck's theory.

Strehler: What is the cross section? Will it be an efficient trap for the energy? It is the total optical cross-sectional overlap, as well as whether it is in the right position, that is important. Can you estimate this?

Linschitz: The quantitative measurements in the far red that we have to date

are very crude, but I'd estimate that the total absorption in the new band is about a fourth of the red chlorophyll band.

Strehler: One quarter.

Linschitz: Maybe one fourth. You could certainly see it.

Bassham: One thing this whole picture is based on is the assumption that one quantum transfer implies one electron transfer. Many people do not agree with that assumption. I would be interested in some comments in favor of the argument that one cannot transfer one electron by one light quantum. I personally believe that you probably do transfer one electron per quantum. Franck's theory would require, among other things, a minimum quantum requirement of 8, if you have to have two light quanta for each electron transfer.

Rabinowitch: This obviously is not explained in this abstract because it implies, as far as I understand, that you would need to know the energy of the free radical. The transfer of one hydrogen atom creates a free radical. We usually don't know what the energy of this free radical is. Franck says, "The excitation energy of 41 kcal. (first excited singlet state) permits only the transfer to a strong (not a weak) oxidant." That must mean the energy we would assume is needed to transfer one hydrogen or one electron to PGA or to TPN.

Bassham: For example, the energy of 40 kcal. is equivalent to about 1.7 electron volts per electron. This is sufficient to make hydrogen peroxide from water at a concentration of 10^{-8} molar and at the same time to reduce TPN to reduced TPN.

Rabinowitch: You are using the average for the two hydrogens.

Bassham: Well, do we know enough about the actual structure of grana to say that it might not have some type of arrangement of semiconductors which makes possible the separation of these charges, providing the electron is of the right potential at the right place? I think this is certainly within the realm of possibility.

Gaffron: The gist of the entire discussion this morning indicates the singlet state is not directly responsible for the chemical reaction. Thus you cannot work with 41 kcal., you have to lower the available energy to about 30.

Bassham: Even with 33 we can just barely make it.

Rabinowitch: Well, that ignores the need of going over the radical state.

Bassham: Presumably there is some system for getting around the radical state to accomplish this. Reactions that in solution chemistry would require radicals are often accomplished *in vivo* with much lower energy.

Brugger: I should mention that Dr. Franck was certain that there was going to be a discussion of electron transfer here. He definitely advocated hydrogen atom transfer. He simply could not see his way clear to agree to electron transfer in this case because, as has been mentioned, you don't have much time. If one transfers electrons and makes hydrogen ions, he has to hydrate the hydrogen ions, or otherwise there isn't enough energy. He felt that one hasn't enough time to hydrate hydrogen ions and not enough light energy to transfer electrons without hydrating the hydrogen ion.

Bassham: I don't think it takes very long to transfer an electron.

Brugger: You have to pay a penalty. The ionization potential for a hydrogen atom is 13 volts. That is, 300 kcal. are required to make a hydrogen ion and an

electron. The hydrogen ion, i.e., a bare proton, can, however, hydrate to form a hydronium ion. This hydration liberates about 270 kcal. Roughly, one can remove an electron from a hydrogen atom and make a (hydrated) hydrogen ion with 30 to 40 kcal. only if one hydrates the proton. Similar considerations explain why the heat of ionization of water is about 13 kcal.: both the H^+ and the OH^- are hydrated—an exothermic process. Dr. Franck intended to point out that there are no reactions known of the type:

(1) $\text{dye} + h\nu \longrightarrow \text{dye}^*$

(2) $\text{dye}^* + H_2O \longrightarrow \text{dye} + H^+ + OH^-$

where $h\nu$ is 30 to 60 kcal. He also wished to mention the reactions:

(3) $(Fe^{++})_{aq} + h\nu \longrightarrow (Fe^{+++}OH^-)_{aq} + H$

(4) $(Fe^{+++})_{aq} + h\nu \longrightarrow (Fe^{++})_{aq} + H^+ + OH$

The second of these does not proceed using visible radiation because there is not time to hydrate the H^+ during the photochemical act and thus to gain the energy of hydration. A quantum of visible light does not have sufficient energy to liberate the bare, unhydrated proton. The Franck-Condon principle (conservation of momentum) restricts movements of atomic nuclei during photochemical acts. The energy requirement for the reaction liberating a free proton (H^+) is thus very great.

Bassham: The electron is not necessarily taken from a hydrogen atom. It may be taken from water.

Brugger: What difference does it make? You still have a bare proton sitting around which you have to hydrate.

Bassham: Yes, but we can take several electrons at the same time from water, or we can get away from the radical in between. And, since we have an aggregated chlorophyll system where we can have a high population of exited chlorophylls at the same time, it is conceivable that we don't have to form any radicals in between.

Brugger: I don't mean to dodge the issue but I think it would take too long to go into it. Dr. Franck's theory was specifically designed to provide a mechanism whereby hydrogen atoms could be transferred.

Rabinowitch: Whenever you transfer electrons, you immediately create an uncomfortable position which requires a subsequent adjustment of the nuclei to fit the new distribution of charges and you lose some of the energy. In other words, electron transfer requires a high activation energy. It is better to transfer hydrogen atoms with only the energy needed for the transfer and without extra energy of activation, whther it is one or two atoms at a time.

Brugger: I can make one more point while I think of it. Often the electron transfer reactions involve two electrons at a time. The energy is calculated per electron by just dividing the energy by two. One has absolutely no guarantee that the energy required to transfer the first electron is half the sum required to transfer both. So if you can just squeeze by theoretically in transferring one electron at half the energy of the two-electron process, you have no guarantee that this actually works.

Bassham: We are not working with one atom or one molecule at a time. If we accumulate charge at a certain potential, which is necessary, say, for the DPN electrode, that is all we have to accomplish. There can be a large population of electrons.

I might point out that in something like a solar battery, for instance, you can remove an electron from one position. It will by conduction fall into a hole somewhere. This creates a charge. The charge can be used for the chemical current or wherever you want to use it. It may be the same in photosynthesis.

Strehler: There are some energetic considerations here that Dr. Bassham is not taking into consideration—at least not here. (*1*) Energy will be dissipated (and a fair amount of it) in the process of oxygen liberation (I assume we're speaking about green plants). (*2*) In all likelihood that energy has to be available in addition to the energy dissipated to form a triplet state or to store the usable energy in some stabilized pool or intermediate reductant. If this were not the case the reductant could react back at high rate. (*3*) In addition, not all of the energy in the one electron carrier or free radical may be available to do chemistry since radicals generally dismutate to nonradicals with a loss of energy—and DPNH is a radical.

I don't believe it is possible at present to formulate binding arguments of a quantitative sort in support of either position. But the arguments raised on purely physical chemical bases, such as given by Franck in his review paper in the *Archives of Biochemistry and Biophysics*, or as presented in the Phosphorus Symposium, where I developed essentially similar conclusions on entirely independent basis, cannot simply be dismissed. We must be seriously concerned about the question: Can one quantum of 40 kcal. energy content, considering probable losses, transfer an electron from the potential of water to the potential of an acceptor which reacts reversibly with fixed carbon dioxide?

Bassham: Take 41 kcal. and throw away 7 or 8 kcal. in the first photochemical step to make a triplet state. Then you still have enough left, as I said before, to create hydrogen peroxide and to reduce TPN^+ to TPNH. Then the hydrogen peroxide proceeds spontaneously to water and oxygen.

Strehler: How do you make the hydrogen peroxide? By the recombination of OH radicals?

Bassham: We don't necessarily go through the OH radical. Perhaps we have a hydrated surface from which we have enough electrons all at one time to allow instantaneous formation of peroxide. I am simply saying that we don't know enough about it to rule out any possibility as long as there is enough energy to carry out the net reaction which is there.

Rabinowitch: The free energy of photosynthesis is approximately 120 kcal., or about 30 kcal. per hydrogen atom transferred.

If you want to transfer these four hydrogens by four protons from water to something of the same reduction potential as your ultimate acceptor, which is what you want to do in the case of TPN, you have practically nothing to spare.

Bassham: I think you misunderstand me. I am not proposing that four quanta will accomplish photosynthesis. I say that we can transfer four electrons. Other electrons have to be used up to make high-energy phosphate to help us out later on. To do what you are suggesting would require a better reducing agent than

TPN. Actually, we don't get by with just transferring four electrons. We have to transfer six or seven electrons and need six or seven quanta.

Rabinowitch: For that you have to have a process which would liberate energy after these transfers. Do you want to leave this just to the decomposition of hydrogen peroxide?

Bassham: That is spontaneous.

Rabinowitch: It evolves energy. Are you going to salvage this energy somehow?

Bassham: No, we don't need it.

Rabinowitch: For reduced TPN you really need some more energy to shoulder it up.

Bassham: We do get that energy when we burn part of the TPNH oxidatively.

Rabinowitch: All right, so that would take care of itself, but the fundamental process, the transfer from the level of water to hydrogen, i.e., the level of TPNH, requires almost a full quantum itself.

Duysens: In one absorbed quantum you have 40 kcal. Of course, a certain amount is lost to form stable compounds. Let's say 8 kcal. Then you have 32 kcal. left for reduction of DPN. Transfer of one hydrogen from water to DPN requires only 26 kcal. So I think it is possible.

Rabinowitch: You will have just a very little left. It is just barely possible.

Strehler: If you form peroxide as an intermediate you lose another 9 kcal. which are not available for the formation of a potent reductant.

Kamen: This is anticipating the argument that I expect tomorrow. The picture that Dr. Bassham has been giving here is very similar to the one that we have been trying to work out in connection with the interaction between chlorophyll and cytochrome. But my feeling is that this discussion is fruitless and we will get nowhere, for the simple reason we are not dealing with pure chlorophyll in photosynthesis. If you put this chlorophyll molecule on protein, I will venture to say that all these energy requirements will disappear or be changed so that you will have a different picture entirely. Until we find out something about native chlorophyll and about the biological capacity of it, I think we should postpone all energy considerations entirely.

What I am saying is that the cooperative pigment part of the assembly takes up energy which is distributed over a large molecule, the sink of which we don't know. The sink may not be the triplet state at all. It may be a double bond somewhere else. It may very well be (I say this with great hesitation) an iron hematin. These are present in great amounts and can do everything that is needed in photosynthesis, as you will see tomorrow.

There are so many processes that are impossible from a strictly physicochemical standpoint but which occur all the time in biological systems, that the argument that something cannot happen because 7 or 8 kcal. are lost is just plain senseless. I think the best thing to do is to table the whole argument and not waste any more time.

Brugger: Dr. Franck, I think, certainly never denied any electron transfer processes, but I think he felt if you used photochemistry to move electrons around you had to pay some price. Since you had to do it in a short time you had to use

more energy. It certainly is not the most economical way to do the process. He did not deny electron transfer and he certainly believes in semiconductors.

If you want to do this efficiently with chlorophyll molecules, as Dr. Rabinowitch pointed out, you also have to do some moving around and you cannot do this in 10^{-12} second. If you have lots of energy, if you use gamma rays, then you can most likely do it. With 30 electron volts per ion pair, you can do it that fast. If you have 800 or 900 cal., you have plenty to work with.

Rabinowitch: This ring V seems to be the key chemically, or maybe it is a sort of general self-hypnosis we are indulging in, feeling that there must be something in this ring which would explain the participation of chlorophyll in photosynthesis. Anyhow, it seems also to be significant for the infrared absorption spectrum of chlorophyll. All the changes and variations of chlorophyll which have been revealed by the visible absorption spectrum also must find their counterpart in the changes in the infrared spectrum. Dr. Holt is going to say something about the infrared spectrum as a means to understanding this structure of chlorophyll and particularly what happens in this one ring.

Reversible Spectral Changes in Chlorophyll Solutions, Following Flash Illumination*

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Theories of the mechanism of dye-photosensitized reactions, in particular those catalyzed by chlorophyll, have long postulated the intermediacy of metastable excited states of the dye. This assumption was based originally on indirect evidence, such as the maintenance of high photochemical quantum yields even at low substrate concentrations, and the lack of fluorescence quenching by photochemically efficient substrates (1). More recently, direct verification of the existence of such long-lived states has been provided by study of reversible spectral changes in chlorophyll solutions, either following intense flash irradiation (2–4) or under steady cross illumination in rigid solvents, under conditions precluding bimolecular solute reactions (5). In this report we present observations and remarks on flash-bleaching of chlorophyll in fluid solvents. The chief results to date are the verification of the general shape of the transient spectra, observation of a new far red absorption band in the "metastable state," and demonstration of kinetic effects of polar and nonpolar solvents.

APPARATUS

This is shown schematically in Fig. 1. A beam of light from source "*S*" was collimated by lens "*L*," passed through a shutter, the dye solution, thence through a monochromator and onto a photomultiplier tube. Light transmissions at selected wavelengths were recorded on the oscillograph, immediately preceding and following flash excitation.

A major problem in using this technique is to prevent swamping of the measuring light by scattered light from the flash. Sample fluorescence may also interfere. Toward this end, the monochromator was

* Research performed under the auspices of the U. S. Atomic Energy Commission.

placed in the optical train *behind* the flash tube-cell assembly, and the distance between the sample cell and entrance slit of the monochromator was made large (120 cm.) to favor the collimated beam. Scattered light was further decreased by passing the measuring beam through accurately aligned apertures in a series of baffles placed in a light-tight box, completely enclosing the optical path. The whole assembly was mounted on an optical bench. Steady-state bleaching due to the undispersed measuring beam was negligible, compared to that caused by the flash. Provision was made for inserting filters in the beam to

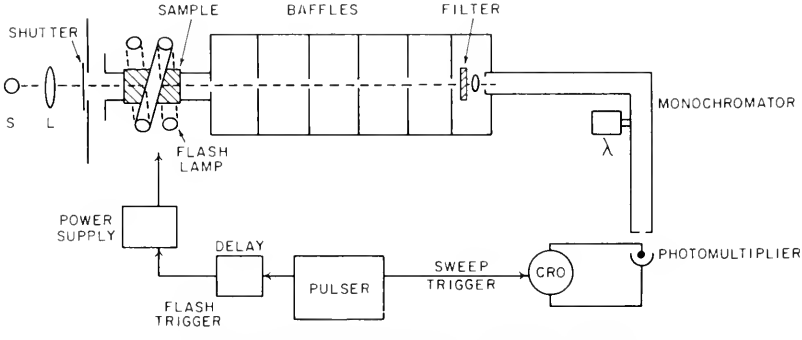

Fig. 1. Apparatus for flash-illumination studies.

improve the purity of the spectrum provided by the monochromator (a constant-deviation Gaertner instrument). The source "*S*" was a 500-watt concentrated filament projection lamp, operated from a stabilized d.-c. supply. The sample cell, 50 mm. long and 13 mm. I.D., carried a side ampoule permitting solutions to be prepared and degassed on the vacuum line, and sealed off. The xenon-filled flash tube was made of 17-mm. Pyrex tubing, wound in a two-turn helix, within which the sample cell was mounted, the whole being surrounded by a cylindrical magnesia-coated reflector. The tube was operated at 4000 volts and 24 mfd., and provided a flash of about 100 microseconds duration. A pulse generator and associated circuitry was used to trigger the sweep of the oscillograph, and after an adjustable delay, to fire the flash tube.

PROCEDURE

In order to correct for scattered or fluorescent light the following measuring procedure was used. With the shutter *open*, and sample in

position, the monochromator slits and amplifier gain were adjusted to give a suitable deflection on the screen at the desired wavelength. The tube was then flashed and a sweep record obtained in which the vertical deflection represents the total of transmitted *and* scattered light reaching the photocell. The shutter was then *closed*, and a second flash and sweep recorded, giving just the scattered light. The vertical distance between the two traces then measures the net transmitted light at the selected wavelength and time. A timing sweep, blanked at 10,000 cycles by a calibrated audio oscillator, was also taken for each measurement. Oscillograph deflections were converted to optical densities by calibration records, using either pure solvent or the measured absorption spectra of the test solutions. Linearity of the photometric system was checked occasionally with a calibrated screen.

RESULTS

Typical results, showing bleaching at the red peak and the appearance of new absorption bands in the far red and green, are presented in Fig. 2. It is obvious that flash-illumination leads to drastic changes in absorption spectrum, confirming at least qualitatively the spectrographic observations of Livingston *et al.* Systematic measurements at various monochromator settings enable one to obtain the spectrum of the metastable species. Our data are in general agreement with those of Livingston and co-workers in showing a decreased absorption at the red and blue peaks of chlorophyll and enhanced absorption in the green (around 525 mμ) and violet. Of particular interest, however, is the new band in the far red ($\sim$700 mμ), demonstrated in Fig. 2B. It is probable that this is the same band first seen in the low-temperature steady-state bleaching experiments, in rigid solvents (5). However, the lifetime measurement afforded by the flash technique permits us now to assign the far red absorption unequivocally to a metastable species.

The existence of a reasonably intense far red band in the metastable state has been predicted by Franck on the basis of a postulated structural analogy between the enolate ion and triplet state of chlorophyll (6,7). In his most recent picture of the mechanism of splitting of water by excited chlorophyll, discussed elsewhere in this volume, Professor Franck has also suggested that the function of this band is to enable chlorophyll, in its metastable form, to become still further

excited by resonative transfer of energy from the rest of the chlorophyll aggregate (6). The flash data show that, in agreement with Franck's postulate, the metastable form of chlorophyll does indeed have an absorption band strategically placed to permit trapping of

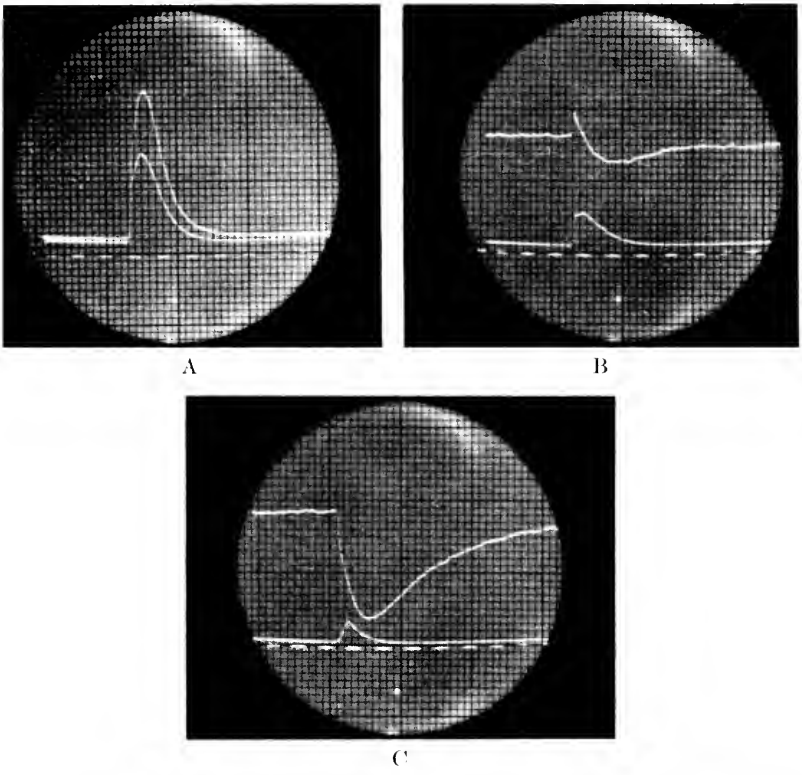

Fig. 2. Changes in light transmission through chlorophyll-*b* solutions, following flash illumination. Solvent, deoxygenated 95% methanol; concentration, 1.2×10^{-5} *M*; upper sweep, flash plus measuring light; middle sweep, flash alone (base line); lower sweep, 10,000 cycle time marker. A, 655 mμ (red peak); B, 700 mμ; C, 525 mμ.

energy from the first excited singlet levels of adjacent molecules. It is of interest to point out that other "reversibly bleached" chlorophylls, such as the phenylhydrazine reduction product, do *not* show any appreciable absorption beyond the original red peaks.

The curves given here are taken with considerably better signal-to-

noise ratio and more nearly monochromatic scanning light than was used by Livingston and Ryan in their original flash experiments (2). In addition, the present technique permits determination of the zero-time of the flash and thereby observation of the development as well as decay of the spectral changes. Despite these improvements, definitive kinetics are still difficult to obtain, essentially because the flash duration is comparable to the lifetime of the excited species. This limits the experimental accuracy that can be achieved (transmission changes are obtained by difference) and greatly complicates the theoretical treatment. Our apparatus is now being modified to provide much shorter flashes.

It has been suggested that a dye-solvent redox reaction might be the mechanism of formation of a metastable species (1,5), and the strong solvation reactions of chlorophyll (8–10) are consistent with this view. To clarify the question, flash experiments were carried out in purified methylcyclohexane, exhaustively dried by distilling over calcium hydride, and then pumping away a large fraction of the solvent at low pressure. The extent of dehydration was indicated by the drop in fluorescence of the resulting chlorophyll *b* solution to 8% of its original value in the undried solvent (8) and the appearance in the dry solution of a new band at 665 mμ, about equal in intensity to the usual "wet" band at 655 mμ (9). On flashing, the "dry" band at 665 mμ was found to bleach very markedly. The strong bleaching in completely nonpolar solvent, as well as the appearance of a transient spectrum rather similar to that observed in methanol or pyridine, implies that at least one of the metastable states is reached from the excited singlet by an intramolecular process alone. Pheophytin and zinc tetraphenyl porphine also show marked spectral changes after flash illumination.

In pure hydrocarbon solvent, the appearance and decay of absorption at 525 mμ occurs at the same rate as the bleaching out and recovery of the 665-mμ band, the situation apparently being simpler than in polar solvents (4). The reactions in the polar case thus may involve desolvations or keto-enol shifts in the excited molecule, or excited dye-solvent reactions following formation of the first metastable product. In this connection, it is of interest that in pyridine, the half-life of the 525 absorption is considerably increased, and the reversibility of the reaction is much better than in carefully purified 95% methanol.

Further study of the kinetics, using flashes of much shorter duration, is clearly required.

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Infrared Spectroscopy of Chlorophyll and Derivatives

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Several reversible chemical and photochemical reactions of chlorophyll *in vitro* are known (1–3), but little is known about the groups in the molecule which are involved in these reactions. The study summarized below, and presented in detail elsewhere (4), was undertaken in the hope that infrared absorption data may give specific answers. Various derivatives of chlorophyll and ethyl chlorophyllide have been prepared to permit unequivocal assignment of the absorption bands. Of the atomic groups present in the chlorophyll molecule, only the C=O, C=C, O—H, N—H, and C—H groups gave infrared absorption bands which could be assigned with reasonable certainty. These bands lie between 3600 and 1600 cm.$^{-1}$; the spectrum below 1600 cm.$^{-1}$ proved too complex for definite interpretation.

Table I and Fig. 1 summarize the data. Of the three C=O groups present in pheophytin *a* and ethyl pheophorbide *a*, the ester groups at C_7 and C_{10} absorb near 1740 cm.$^{-1}$, which is the usual frequency found for the C=O group in an ester (5). The keto group of the cyclopentanone ring absorbs at 1697 cm.$^{-1}$ (in $CHCl_3$-solution) or 1706 cm.$^{-1}$ (in CCl_4-solution). This agrees with the assignment given to this band in the earlier work of Weigl and Livingston (6). The decline of the frequency from 1740 cm.$^{-1}$, the absorption frequency of the keto group of cyclopentanone itself, to about 1700 cm.$^{-1}$ must be due to the attachment of the isocyclic ring to the aromatic nucleus. That the 1697 cm.$^{-1}$ band is in fact due to the keto group of the cyclopentanone ring is indicated by its absence from the spectrum of the oxime of ethyl pheophorbide *a*, and from those of the 2,4-dinitrophenylhydrazine derivatives of pheophorbide *a* and pyropheophorbide *a*. The C=O in the carboxyl group of the propionic side chain in position 7, present in the two latter compounds, absorbs at 1705 to 1706 cm.$^{-1}$, and thus

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TABLE I. Infrared Absorption Bands of Chlorophylls and Derivatives. Frequencies Corrected (cm.$^{-1}$)

Compound	Solvent	NH	OH	C—H phytol	C—H Aldehyde	C=O					C=C
						Ester	Ketone	Carboxyl C_7	Aldehyde	Chelate	
Ethyl chlorophyllide *a*	$CHCl_3$	—	—	—	—	1733	1678	—	—	—	1610
Chlorophyll *a*	Crystals in Nujol	3605?	3440+ 3252	2920	—	1727+ 1740	1694	—	—	1640	1604
Chlorophyll *a*	CCl_4, CS_2	—	3350 (weak)	2916	—	1735	1691	—	—	1652	1610
Chlorophyll *a*	Ethyl ether	—	—	—	—	1738	1698	—	—	—	1608
Chlorophyll *a*	Pyridine	—	3400 (strong)	—	—	1740	1683	—	—	—	—
Ethyl chlorophyllide *b*	$CHCl_3$	—	—	—	2720	1730	1689	—	1657	—	1610
Chlorophyll *b*	$CHCl_3$	—	—	2920	2720	1728	1688	—	1655	—	1610
Chlorophyll *b*	Crystals in Nujol	—	3360	2924	2710	1738	1697	—	1655	—	1607
Chlorophyll *b*	CCl_4, CS_2	—	3350	2924	2710	1738	1701	—	1664	—	1609
Pheophytin *a*	CCl_4	3392	—	2930	—	1740	1705	—	—	—	1618
Pheophytin *a*	Pyridine	3382	3100–3700	2920	—	1736	1700	—	—	—	1617

Ethyl pheophorbide *a*	CS_2	3375	—	—	—	1743	1706	—	—	—	1618
Ethyl pheophorbide *a*	$CHCl_3$	3380	—	—	—	1735	1697	—	—	—	1620
Pheophorbide *a*	$CHCl_3$	—	2400–2800	—	—	C_{10}: 1740	1702	1702	—	—	1622
Pheophorbide *a*-2,4-dinitrophenyl-hydrazone	$CHCl_3$	—	—	—	—	C_{10}: 1728	—	1705	—	—	1613
Pyropheophorbide *a*	$CHCl_3$	—	—	—	—	—	1685	1704	—	—	1620
Pyropheophorbide *a*-2,4-dinitrophenyl-hydrazone	Nujol mull	—	—	—	—	—	—	1725	—	—	1610
Pheophytin *b*	CCl_4	3410	—	2930	2720	1740	1709	—	1665	—	1618
Ethyl pheophorbide *b*	$CHCl_3$	3380	—	—	2720	1737	1704	—	1663	—	1620
Ethylpheophorbide *b*-2,4-dinitrophenyl-hydrazone	$CHCl_3$	—	—	—	—	1740	1710	—	—	—	1618
						Ester		Ketone	Acetyl		
Bacteriochlorophyll	CCl_4	—	3350	2916	—	1735		1683	1655		1640
Methyl bacteriochlorophyllide	$CHCl_3$	—	—	—	—	1727		1662	1662		1640

adds some confusion to the interpretation of the spectra, since it is superimposed on the absorption band of the keto group at C_9.

We assign the band at 1615 cm.$^{-1}$ to the C=C bonds in the aromatic nucleus; this assignment is supported by the strong absorption shown in the same region by the phenylhydrazine derivatives and by the presence of a similar band in the spectrum of bacteriochlorophyll, which contains no vinyl C=C group.

The spectra of chlorophyll *a* and ethyl chlorophyllide *a* show marked differences, in the region of the C=O bands, from those of the magnesium-free derivatives. These depend on the choice of the solvent. Spectra taken in nonpolar solvents (CCl_4 or CS_2) or with crystals of chlorophyll *a* dispersed in Nujol (mineral oil), show an extra band at 1640 to 1650 cm.$^{-1}$. This band is interpreted by us as indicating a strongly hydrogen-bonded (internally chelated) form of the β-keto ester in ring V. In polar solvents, e.g., diethyl ether or chloroform, chlorophyll *a* does not show any such band.

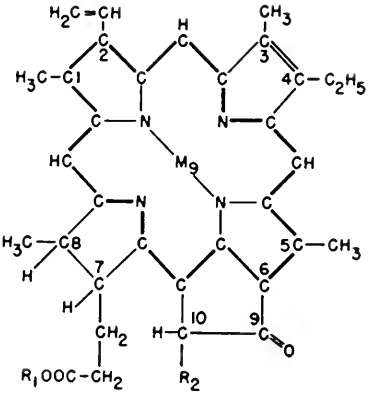
Spectra of pheophytin *a* in pyridine show a band characteristic of a hydrogen-bonded hydroxyl group, between 3100 and 3700 cm.$^{-1}$. We believe this could be due to enolization of the β-keto ester, in position 9, and hydrogen bonding between the pyridine nitrogen atom and the enolic O—H group.

The aldehyde group of chlorophyll *b* (or pheophytin *b*) absorbs at 1663 to 1665 cm.$^{-1}$; this band is absent from the spectrum of pheophytin *a* and from that of the 2,4-dinitrophenylhydrazine derivative of ethyl pheophorbide *b*.

Spectra of chlorophyll *b*, taken in CS_2 or CCl_4 solutions and with crystals of chlorophyll *b* dispersed in Nujol, do not—in contrast to those of chlorophyll *a*—show a band indicating the occurrence of the chelated enol form of the β-keto ester in ring V.

The absence of this band from the spectra of chlorophyll *b* must be due to the presence of the oxygen atom in the formyl group in ring II. This electronegative group in position 3 may neutralize the tendency of magnesium to shift negative charge to the oxygen in the keto group in position 9, and thus reduce the tendency of the β-keto ester for enolization.

To summarize: infrared spectra indicate that in the crystalline state and in nonpolar solvents the β-keto ester group of chlorophyll *a* is present as a chelated enol. In polar solvents, such as chloroform or diethyl ether, there is no indication of similar enolization. Chlorophyll *b*,



Compound	Mg	R_1	R_2
Chlorophyll *a*	+	$C_{20}H_{39}$	$-C(=O)OCH_3$
Pheophytin *a*	−	$C_{20}H_{39}$	$-C(=O)OCH_3$
Ethyl chlorophyllide *a*	+	C_2H_5	$-C(=O)OCH_3$
Ethyl pheophorbide *a*	−	C_2H_5	$-C(=O)OCH_3$
Pheophorbide *a*	−	H	$-C(=O)OCH_3$
Pyropheophorbide *a*	−	H	H
b Compounds of the *b* series same as above except for replacement of methyl group in position 3 by $-C(H)=O$			
Bacteriochlorophyll*	+	$C_{20}H_{39}$	$-C(=O)O-CH_3$
Methyl bacteriochlorophyllide	+	CH_3	$-C(=O)O-CH_3$

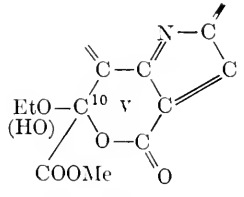
* Differs from chlorophyll *a* by vinyl group in position 2 being replaced by $-C(=O)CH_3$, and the double bond $\Delta^{3,4}$ saturated.

Fig. 1.

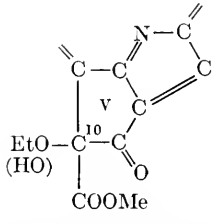
whether in the crystalline state or in polar or nonpolar solvents, gives no evidence, in its infrared absorption spectra, of the formation of an enol. From methanolic solutions exposed to air, several products of allomerization of chlorophyll *a* were separated chromatographically. Comparison of visible absorption spectra, acid numbers, and other properties of these products and their pheophorbides with those of compounds characterized earlier by Fischer (7) shows them to be phyllins of (*1*) purpurin-7-triester, (*2*) purpurin-7-lactone ether, (*3*) 10-oxy-pheophorbide *a*, and (*4*) purpurin 18. The infrared spectra of the phyllins of (*2*) and (*4*) in CCl_4 are difficult to reconcile with the known chemical structural formulas, and are markedly changed upon removal of magnesium. The phyllins of (*1*) and (*3*) readily form the "Krasnovskii reaction" intermediate; all four products are bleached by $FeCl_3$ in methanol solution.

Discussion

Linschitz: A study of the allomerization reaction has recently been made in our laboratory by Mr. Sol Gilman, who has obtained data on the nature of the different allomerization products which nicely supplement Dr. Holt's very thorough infrared study. In particular, the results indicate, in agreement with Dr. Holt's proposals (and earlier work as well) that his Fraction-2 is a chlorophyll oxidation product with a lactone structure in ring-5 (A), while Fraction-3 is a similar product but *without* the lactone oxygen (B).



A ("Fraction = 2," Holt)
(α = ethoxy, α' = hydroxy)



B ("Fraction = 3," Holt)
(β = ethoxy, β' = hydroxy)

In Gilman's work, methods were developed for preparing each of these four derivatives separately in good purity and yield. For example α' can be made by exposing the enolate of chlorophyll to air, in pyridine solution, β' by allomerizing chlorophyll in alcohol with ammonium acetate, and β by the oxidation of chlorophyll with quinone, in air-free alcohol. The structures were established by converting to previously known compounds, by chemical analysis, and by spectral and chromatographic data. The derivatives α, α', and β' constitute about 90% of the various products obtained when chlorophyll is allomerized in the usual way,

by standing in alcohol. The main product (about 60%) is, of course, α. The infrared spectra which we have on these compounds check those of Dr. Holt, and his interpretations are in complete agreement with our chemical evidence.

Some observations on the visible spectra of these derivatives may be of interest. The simple replacement of C_{10} hydrogen by hydroxy or ethoxy, giving a type "B" compound, has very little effect on the chlorophyll spectrum. However, formation of a type "A" compound by insertion of the lactone oxygen into ring V removes the high-energy shoulder from the Soret band of chlorophyll, and shifts the whole spectrum to higher energies. Replacement of ethoxy by hydroxy on C_{10} has no effect whatever in either type "A" or type "B" spectra.

Aronoff: What I find difficult to conceive is the change in the capacity for hydrogen bonding simply because of the presence or absence of magnesium. I wonder if the mechanism could not possibly involve a racemization of hydrogen at C_{10} (which, of course, is optically active).

Rabinowitch: It is well known that the presence of magnesium in the center affects the far corners of the chlorophyll molecule. I think if you have a positive charge in the center it polarizes the ring system, with the outside becoming positive, and this favors enolization of ring V. In a *b* compound, there is a second "sink" at another corner of the ring system; consequently, less positive charge accumulates in ring V.

It may be collective self-deception, but there is general feeling that there is something important about the lower right corner in the chlorophyll molecule. We ask: why is it chlorophyll *a* that plays the star role in photosynthesis? Perhaps, you get the proper capacity for reversible changes in ring V by having magnesium in the center, and you take away some of this capacity by having a CO group in ring II. It seems suggestive.

Weigl: I am a little puzzled about the chlorophyll *a* chelate. Is there a possibility that you had dry carbon tetrachloride in the study of chlorophyll *a* and a not-quite-as-dry solvent in the case of chlorophyll *b*, or vice versa?

Holt: No! In the chlorophyll *b* spectrum, there was definitely an OH band, a strong "associated OH" band.

Becker: You say chlorophyll *b* shows no chelation?

Holt: Yes.

Becker: And the chlorophyll *a* showed it definitely?

Holt: It showed an indication of a chelated C—O-group bonded to the ester. Chlorophyll *b* did not.

It definitely looks as though water is incorporated into chlorophyll *b* as the OH band is always there.

Smith: There is a great difference in the ease with which you can pull the magnesium out of *a* and *b*. It takes 35% acid to pull it out of *b*, while 25% acid is enough to pull it out of *a*. In other words, magnesium seems much more strongly bonded in *b* than in *a*. Whether this has anything to do with the difference in chelation, I don't know, but it seems a possibility.

Rabinowitch: I don't know whether we have a theoretical organic chemist here who could say whether it is plausible that a magnesium atom in the center of a molecule would affect the tendency for enolization in ring V. Dr. Becker?

Becker: I made the point this morning—and everything since then has borne it

it out—that magnesium produces a perturbation of the whole system.

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Dark- and Light-Activated Chemiluminescence of Chlorophyll *in Vitro*

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The possibility that the light emitted by green plants after they are illuminated is a decay from some metastable state rather than due to a chemical reaction has been considered by various workers in the field. Some time ago an attempt was made to determine whether chlorophyll adsorbed to proteins will phosphoresce after illumination. Under no conditions was any phosphorescence by artificial chlorophyll-protein mixtures or complexes observed. Indeed, the loss of luminescence ability by chloroplasts even when stored at −20°C. strongly suggests that phosphorescence from a metastable state is not involved since the physical properties of the chlorophyll-protein complex should not be drastically altered under these conditions of storage (1,2).

Further independent evidence suggesting that chlorophyll can be excited to luminescence by a chemical reaction or reactions *in vivo* was furnished by the present findings that chlorophyll solutions will, under certain conditions, chemiluminesce.

During the last year we have attempted to measure the effect of illumination on the concentration of reduced coenzyme I in leaves and algae (3). As an assay system we used the response of the bacterial luminescent system to added DPNH (4). This assay system contains as an activator of the bacterial system, long-chain aldehydes (decyl aldehyde, in this case) (5). Upon mixing solutions obtained from algae extracted with hot alkali we observed a fairly bright luminescence with the "quantum counter" which depended on the prior illumination history of the plants from which it was obtained. However, on closer examination, it was found that the luminescence occurred even in the absence of the bacterial enzyme and that the light emitted was red in color.

We have undertaken the purification of the material in the alkaline plant extract and have found that it was a chlorophyll *a* derivative. Subsequently "pure" ethyl chlorophyllide and chlorophyll *a* were shown to emit a fairly bright light in the presence of base, oxygen, and aldehyde in organic solution. Moreover, the intensity of the luminescence was increased if the solution was illuminated before measurement.

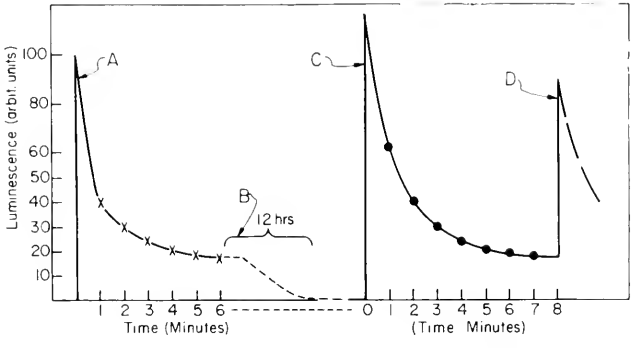


Fig. 1. Time course of luminescence of chlorophyll in methanol (with base and aldehyde). A, chlorophyll in ether added to basic methanol plus formaldehyde. B, solution kept in dark for 12 hours, at the end of which no luminescence was observable. C, sample illuminated after 12-hour dark period. D, sample illuminated again. T = 25°C.; 0.1 ml. 0.01 M NaOH in methanol added to 0.5 ml. methanol containing 0.01 ml. 25% formaldehyde; 0.1 ml. chlorophyll solution added o.d. at 665 mμ, 5 at 666 mμ.

This suggests that photooxidation reactions are probably involved. Figure 1 illustrates the decay of chemiluminescence and the effect of illumination on chlorophyll luminescence *in vivo*.

The evidence indicates that the light-activated luminescence does *not* require the intactness of chlorophyll ring V (6). After the initial luminescence due to chlorophyll *a* in basic aldehydic solution disappears completely, a brief exposure to light promotes a reappearance of the luminescence even after several days in the dark in base. Under these alkaline conditions the V ring can be presumed to have been hydrolyzed.

Certain changes in the absorption spectrum of chlorophyll take place during the light emission process. Experiments done in collabora-

tion with Dr. James H. C. Smith of the Carnegie Institution of Washington indicate that these changes in absorption are catalyzed by the presence of minute quantities of aldehyde. The changes are illustrated in Fig. 2. Attempts to isolate the newly formed colored compound(s?) have thus far been unsuccessful. A number of products derived from chlorophyll were separated by column chromatography, but the

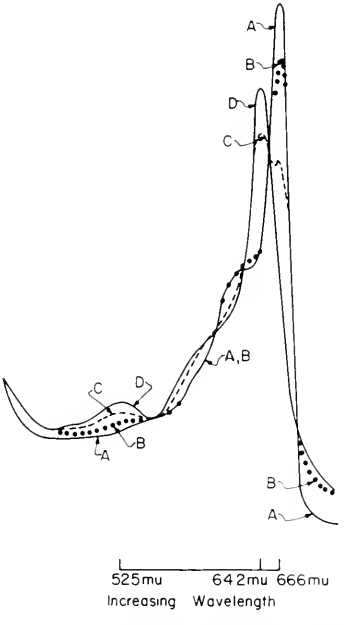
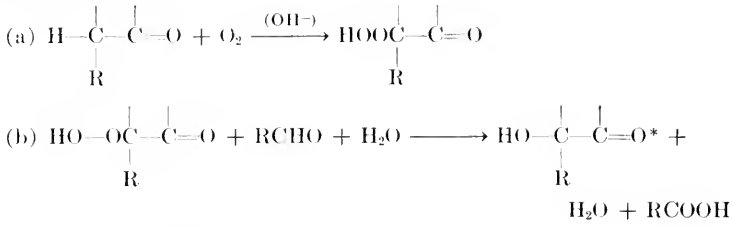


Fig. 2. Spectral changes during the luminescent reaction. Curve A, original solution—chlorophyll in basic methanol. Curve B, after 9 minutes in base. Curve C, 2 minutes after adding formaldehyde. Curve D, 25 minutes after adding formaldehyde. Concentrations as in Fig. 1.

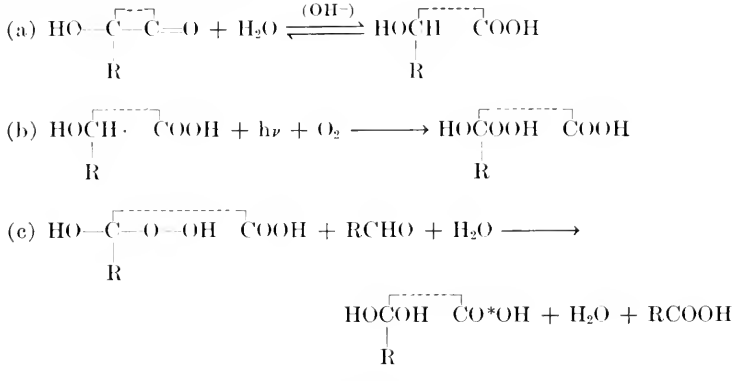
quantities were too small to permit identification of their demagnesiated forms, and it is uncertain whether the several products are the result of the luminescent reaction or of the subsequent treatment of the pigments.

A possible mechanism for these chemiluminescences consistent with the present facts and the known chemistry of chlorophyll is the following:

(1) Dark Reaction (6):



(2) Light-Activated Reaction:



It cannot at present be stated whether these chemiluminescences bear a direct relationship to the process *in vivo*. Such a relationship, if it exists, could be established through the isolation of identical intermediates in the two processes. Possibly, these findings may be of interest only from the standpoint of chlorophyll chemistry. But they certainly lend support to the hypothesis that chlorophyll can be directly excited by reactions in which it is involved or which take place in its vicinity.

Acknowledgments. Thanks are due to Dr. Hans Gaffron and Dr. James Smith for stimulating discussions of some of the results here presented, and to Mr. Charles Soderquist and Mr. John Hanacek for invaluable assistance in the construction of apparatus.

This work was supported in part by a grant from the U.S. Atomic Energy Commission.

Discussion

Lumry: Did you get the emission spectrum?

Strehler: The emission spectrum is very close to that of chlorophyll. It may

be shifted perhaps 10 to 15 mμ toward the blue, and that would fit with this shift observed here in the absorption spectrum changes, but I think better measurements have to be made before one can say anything conclusive about it.

Gaffron: The importance of these observations is not only that they permit an evaluation of the reactions of chloroplasts but that they demonstrate how aggregates of chlorophyll seem to be particularly apt to bind substances which, when they react chemically, may cause chemiluminescence. An oxidation apparently must occur right at the chlorophyll.

Strehler: I should point out that dried chloroplasts treated with ethanol and chloroplasts treated with aldehyde and base do not show this luminescence. You have to remove the chlorophyll from its bound form, I believe, in order to obtain this chemiluminescence.

Gaffron: That would be understandable. In chloroplasts so many other substances which can't be easily oxidized stick to chlorophyll.

Frenkel: What has happened to your efforts to find coenzymes? Is there any chance that you can modify your procedure and use this method for picking up coenzymes? Do you think you can now eliminate that?

Strehler: Yes, I think it can be eliminated. It is a relatively sensitive method, but not as good as fireflies for ATP, and we hope to return to these experiments some time, if somebody does not do them in the meantime.

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Photoreduction of Synthetic Dyes

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The work in our laboratory has been concerned mainly with studies of the kinetics of the photoreduction of synthetic dyes in aqueous media using visible light as the source of energy. The energies associated with visible light vary between 2 and 3 electron volts. It would be useful if this energy could be utilized to bring about the reduction of noncolored molecules as well, the dye serving as the receptor of light. In some cases, we have been able to utilize a large fraction of the absorbed energy for this purpose. I wish to summarize for you the results obtained so far in the hope that they may be of help in understanding the photochemical steps in photosynthesis.

PHOTOREDUCTIONS

It has long been appreciated that some reactions which involve colored substances and which are thermodynamically impossible will proceed, however, in the presence of visible light. For example, Rabinowitch (1) has shown that thionine in the presence of acidified ferrous sulfate is photoreduced to give the leuco (colorless) dye, although in the absence of light this reaction is thermodynamically impossible. In this reaction, the ferric ion which is produced oxidizes the leuco dye back to the colored form and a steady state is reached. We have found that a wide variety of other electron donors such as ascorbic acid, glutathione, cysteine, thiourea, allyl thiourea, and stannous chloride under pH conditions where thionine (or methylene blue) is not reduced in the dark will give a stable leuco dye on irradiation with visible light if oxygen is rigorously excluded (2). The leuco dye exhibits a strong yellow fluorescence (phosphorescence in highly viscous media) and has the property of being oxidized to the normal dye even in the absence of oxygen if irradiated with near ultraviolet light. The fading with visible light, recovery with ultraviolet light, fading with visible light, and so on can be repeated several times and is reminiscent of certain biological processes (e.g., photoperiodism

and photoreactivation) where reversals take place on using light of two different wavelengths.

A particularly interesting electron donor is the chelating agent, ethylene diamine tetracetic acid (EDTA). Nickerson and Merkel (3) have shown that certain dyes in the presence of this substance are photoreduced by visible light. We have examined the kinetics of reduction of methylene blue in the presence of EDTA and found that the quantum yield varies with pH in the same way as does its degree of ionization and its chelating power (4). The leuco dye, in this case, is not fluorescent and does not revert to the normal form on irradiation with ultraviolet light. As with all leuco dyes, however, the normal dye is obtained by flushing the solution with oxygen. EDTA is by no means a reducing agent in the ordinary sense. For example, we found that we could effect the photo-reduction of methylene blue in the presence of EDTA even when a ten thousand-fold excess of hydrogen peroxide is present in the solution. We are now engaged in a more extensive study of the nature of transfer of electrons in photochemical reactions involving EDTA.

The leuco dye is itself a reducing agent and can reduce other substances. For example, leuco methylene blue will reduce tetrazolium salts to their corresponding insoluble formazans (compare refs. 3a and 5). The reduction potential of leuco methylene blue is about zero and it is therefore not a powerful reducing agent. Leuco acriflavine (produced, for example, by photoreduction of acriflavine in the presence of EDTA) is, on the other hand, a very powerful reducing agent (reduction potential about -1 volt) and, if properly utilized, it should be more effective than acidified zinc as a reducing agent. For example, nitrobenzene is reduced by the leuco dye, the latter then reverting to its normal form. Thus, the dye serves as a sensitizer for the reduction of nitrobenzene and is not consumed in the overall process. If the leuco dye has a sufficiently low reduction potential (about -0.1 volt or less) it will produce free radicals (probably hydroxyl radicals) on reacting with oxygen. Thus, if some vinyl monomer is present the chain polymerization of the monomer will ensue, resulting in a high polymeric product (6). Under conditions where chain termination is suppressed the overall quantum yield for monomer conversion may run as high as one billion (7).

LONG-LIVED EXCITED STATES

We have found that very small amounts of certain substances will retard the photoreduction of dyes. For example 2.5×10^{-6} mole per liter of paraphenylenediamine will decrease the rate of photoreduction of eosin (carried out in the presence of allyl thiourea, oxygen being excluded) to one-half its normal value (8). For aqueous solutions at room temperature there are 6.6×10^{9} encounters per second in a liter of molar solution. Hence, we calculate that the lifetime of the photoreactive dye molecules is at least 10^{-4} second. This is about ten thousand times greater than the lifetime of the first excited singlet state. Our studies with several other of the dye-reducing agent combinations show that in all cases the electron donor reacts with dye only when the latter is in the long-lived metastable excited state. Detailed kinetic studies on the photoreduction of fluorescein and its halogenated derivatives (9) show that the steps of the reaction are similar to those proposed for the ethyl chlorophyllide-sensitized oxidation of allyl thiourea reaction by Gaffron (10) and the photobleaching of chlorophyll in methanol by Livingston (11). Our studies show that not only is the quantum yield of photoreduction decreased by the addition of the dye itself but also a wide variety of other dyes (with or without spectra which overlap the spectra of the original dye) will, even in very small concentration, markedly decrease the quantum yield.

The limiting quantum yield obtained by extrapolating the rates to infinite reducing agent concentration varies markedly even with dyes of the same family. For example, fluorescein has a limiting quantum yield of 2.0×10^{-2} whereas for dibromofluorescein the limiting quantum yield is 12.4×10^{-2}. The limiting quantum yield is determined by the relative number of quanta which are used to obtain the long-lived reactive species to those wasted by fluorescence and internal conversion. Results for eosin, for example, show that on an average about 1 of 11 molecules which are in the first excited singlet state undergoes transition to the metastable state. Of these remaining 10 excited molecules about 1.5 (on an average) revert to the ground state with the emission of fluorescence, since the quantum yield of eosin is approximately 0.15, and the remainder fall to the ground state by a radiationless transition.

DYES IN THE BOUND STATE

Most dyes exhibit a spectral shift to long wavelengths when small amounts of a high polymeric material to which the dye binds are added to the solution. In the case of diphenyl and triphenyl methane dyes, the fluorescence of the bound dye is considerably greater than that of the unbound dye (12). Such dyes also exhibit a greater fluorescence when in highly viscous media, and it appears that under such conditions the dye molecules in the excited state spend a longer time in the planar configuration (a necessary condition for fluorescence) than they do in low-viscosity media (13).

Dyes in the bound state have profoundly different photochemical properties from those of free dyes. For example, dyes of the fluorescein family are photooxidized irreversibly with a quantum yield in the neighborhood of 10^{-4} to 10^{-5}. On binding, however, the quantum yield is decreased by a factor of about one thousand. Acriflavine under conditions where it is not photoreduced in the unbound state, is readily photoreduced when bound to polymeric acids (14). Triphenyl methane dyes when bound to polymeric acids resist reduction in the dark on treatment with strong reducing agents which readily reduce the free dye. With light, however, the situation is reversed. Now the bound dye, in the presence of a mild reducing agent, is photoreduced while the unbound dye remains colored (15).

Introduction of certain substances such as nitrobenzene to an aqueous system containing the dye in the bound state and an electron donor inhibits the photoreduction of the dye, the induction period during the irradiation being proportional to the concentration of inhibitor. After the induction period is complete, the rate of the reaction becomes that of the original uninhibited system. It appears that the dye molecules on each polymeric substrate molecule (the local concentration is very high) are acting as a single photochemical unit and a few inhibitor molecules can affect several hundred dye molecules.

Discussion

Rabinowitch: In the case of methylene blue, you also had no quenching of fluorescence?

Oster: No, we used too small amounts of allylthiourea.

Rabinowitch: What is the quantum yield of fluorescence of methylene blue? Ten per cent?

Oster: Much less than 1%.

Wassink: What happens if you leave out the reducing agent?

Oster: Nothing.

Wassink: Do you observe any long-lived species of the dye?

Oster: My only indications are the fading of the dye or the initiation of polymerization. Purely chemical criteria. Gaffron many years ago sensitized the oxidation of allylthiourea in methanol by ethyl chlorophyllide; he was following the oxidation of the reducing agent, while I am following the photoreduction of the dye, by the disappearance of its color.

Gaffron: At that time we could say only that there must be a long-lived state; afterwards it was interpreted as the triplet state of the dye. You need only traces of oxygen for sensitized autoxidation to go with a high yield. This can be understood only if either the sensitizing dye, or all the substrate undergoing oxidation lives long enough in its excited state.

Wassink: In the sensitized reaction, does the sensitizer, chlorophyll itself, change appreciably?

Oster: I think, at least for ethyl chlorophyllide (I cannot speak for chlorophyll), that it is reduced, although I did not see a loss of color. As with the other dyes, the reduced form reduces other substances present. Ethyl chlorophyllide is the only dye which I have studied which does not exhibit a readily discernible loss of color.

Rosenberg: How long is the reduced form of your dyes stable in aqueous systems if you keep out oxidizing agents?

Oster: Leuco methylene blue in solution where oxygen has been rigorously excluded (and no ultraviolet light is present) seems to remain stable forever.

Becker: I would like to point out one important thing. Oxygen in its ground state is paramagnetic, and if oxygen is in intimate contact, so to speak, with the dye molecule, this paramagnetism will allow the triplet state to be occupied to a much higher degree than is allowed ordinarily.

A second point is quenching. Collisional quenching you cannot avoid. You cannot effectively remove electronic energy in small portions unless you have a tremendous number of collisions; so, if you want to remove large amounts of electronic energy, you must have a receiving molecule with an electronic frequency somewhere near the one that is in the donor.

Oster: As a matter of fact, we feel, with no evidence other than what I have indicated here, the reason why certain dyes will inhibit the reaction and others won't, may be that there is a properly situated long-lived (say triplet) state, which we should be able to pick up spectroscopically. That is something we have to look for.

Lumry: Did you try any single-electron reducing agent?

Oster: Chromous chloride is in this category.

Lumry: The organic substances which you have listed as oxidants all require two electrons for their reduction. Some must pass through rather difficult-to-form, one-electron intermediates. Do you believe that two electrons are migrating simultaneously?

Oster: Yes.

Lumry: Glutathione in the absence of oxygen or high hydroxyl concentration

has to go through a radical unless the process involves an improbable activated complex containing two SH-containing molecules.

Hendricks: This discussion is only on the photochemistry of a particular system, but you might be interested to know that the system is a model for the photoreaction of photoperiodism, which is a photoreduction.

Frenkel: What is the maximum energy yield in some of these reactions? I mean, actually is there any conversion of light energy into chemical energy?

Oster: About 10% in the case of methylene blue. My interest is not so much the energy yield as it is the very strong reduction potential of the leuco dye produced. For example, polarographic studies indicate that leuco acriflavine at high pH values has a reduction potential of -1.33 volt.

Weigl: What is the nature of this photoreduced species with its fabulous reducing potential? If it is an excited state of the photoreduced dye, can you get to this state by exciting the photoreduced dye?

Oster: The photoreduced species is the normal leuco dye.

It is a colorless substance. Some of these dyes you cannot reduce in water by any known agent.

Rabinowitch: The difficulty of reducing a substance is not in itself an indication of a lower potential. It may be just an inactive system. It may be a problem of kinetics and not a problem of low potential.

Oster: In the case of acriflavine the indications are that it has a very low reduction potential.

Rabinowitch: Your leuco compound does not reduce everything in the world to carbon.

Kamen: You emphasize this is only the dye-absorbed polymer, not the free dye.

Oster: Not necessarily. For example, acriflavine will not oxidize allylthiourea unless it is bound to the substrate.

Kamen: But you get the same result in the end.

Oster: You get a reduced dye, yes.

Gaffron: Apparently it is still more complicated because you cannot expect that the two hydrogens go over at once. So you must have some radical intermediate.

Linschitz: Under what conditions do your dyes photosensitize polymerization?

Oster: The photoreduced form of the dye in the presence of vinyl monomer is stable as long as oxygen is rigorously excluded. On the introduction of oxygen, however, the monomer is rapidly polymerized.

Linschitz: Hence the photoreduced dye is not a free radical.

Oster: Yes. Free radicals which initiate polymerization are formed during the reaction between the leuco dye and oxygen. The radicals may be OH radicals or the semiquinone form of the dye.

Holt: With ethyl chlorophyllide I tried a system using versene and there is a reversible color change.

Oster: That is very interesting. I want to point out that versene is not what we call a standard reducing agent. As I said before, you can bubble oxygen through it and you don't decompose the material. But it is a reducing agent to light-excited molecules.

Lumry: I want to get this point about Krasnovskii's work clarified if I can.

In his work, is the reduction process of chlorophyll a one-electron or a two-electron process? Does anybody know?

Rabinowitch (*remarks added in proof*): Krasnovskii postulates reduction to a radical (in ionized and neutral form). Linschitz *et al.*, however, found in this system no paramagnetic resonance effects characteristic of free radicals.

Wassink: Have you tried hydrogen gas as a reducing agent for your photoreductions?

Oster: No.

Linschitz: Did you say that the chlorophyllide showed no color change but that it was reduced?

Oster: I said that I could see no color change, and I would like to ask Dr. Gaffron if he saw a color change.

Gaffron: No, but this was in the presence of oxygen.

Oster: In the absence of oxygen I still did not see a color change. I just looked for it by eye.

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Part II

ABSORPTION, SCATTERING, FLUORESCENCE, LUMINESCENCE, AND PRIMARY PHOTOCHEMICAL PROCESS *IN VIVO*

Methods for Measurement and Analysis of Changes in Light Absorption Occurring upon Illumination of Photosynthesizing Organisms

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Upon illumination of photosynthesizing cells a change will occur in the concentration of all intermediates and catalysts participating in photosynthesis. If the changes in concentration result in measurable variations in absorption, these variations at various wavelengths provide information about the kind and function of intermediates and catalysts in photosynthesis.

For colorless cells and their extracts, measurements of spectral changes in absorption of intermediates and catalysts brought about by adding substrates, have proved of utmost importance for the elucidation of biochemical reactions. The magnitude of the change in optical density is proportional to the concentration of cell material. Fairly dense cell suspensions have been used in most experiments. It is not possible, in general, to use concentrations of photosynthesizing cells as high as those of colorless cells, because the photosynthesizing cells are so strongly colored that they would absorb practically all the measuring light. Since the concentration of substances changing their absorption seems to be of the same order of magnitude in photosynthesizing and colorless cells, this may be a reason why, for measurements on photosynthesizing cells, generally an apparatus is required ten to a hundred times more sensitive than the apparatus usable for "colorless" cells. In addition precautions should be taken that the light used for bringing about a change in absorption does not affect the measuring apparatus directly.

APPARATUS AND METHODS FOR MEASURING ABSORPTION CHANGES

The experimental problem is to measure only those variations in intensity of the monochromatic light beam transmitted by a suspension of photosynthesizing cells which are caused by changes in

optical density brought about by illumination. One might try to measure the intensity of the transmitted beam by means of a phototube (or multiplier), amplifier, and meter. However, in many experiments the changes in intensity must be measured with a precision of the order of 0.01%. In order to do this, a meter of great precision is required; and the intensity of the measuring beam, the sensitivity of the phototube (or multiplier), and the amplifier must be constant within 0.01%—requirements which are difficult to fulfill.

Apparatus with compensating beam. The required precision can be achieved by using a compensating light beam from the same light source as the measuring beam. The compensating beam should not change its intensity owing to changes in absorption of the suspensions but should deflect the meter or recorder opposite to that of the measuring beam. If the measuring and compensating beam are of about equal intensity, changes in the electronic parts of the apparatus will not cause marked deflections of the meter; appreciable deflections will occur only if the transmission of the suspension changes. The sensitivity of this device increases with the intensity of the measuring (or compensating) beam.

In the instruments (1,2) used so far successfully, the measuring and compensating beams were alternated with line-frequency by means of mechanical devices, such as moving mirrors, and both beams impinged upon the same multiplier or phototube. The output was passed through an a-c amplifier and caused a deflection of a phase- and line-frequency–sensitive device.

In one type of apparatus (1), the two beams were obtained by splitting the beam leaving the monochromator; the compensating beam bypassed the suspension. A description of such an apparatus will be given below.

In the second type of apparatus (2), the compensating beam is obtained from a second monochromator, and is also passed through the suspension. The wavelength of the compensating beam is fixed and is selected so that no change in absorption upon illumination occurs. The advantages of the latter arrangement are that it compensates to a certain extent for changes in transmission due to sedimentation of the cells, and that it is relatively easy to catch a larger angle of light scattered by the cells. One disadvantage of the second type of apparatus is that it is affected by changes in lamp output, when the wavelengths of measuring and compensating beam are different, since the

emission of tungsten is not the same function of temperature at different wavelengths. It seems also difficult to ascertain whether the absorption of the compensating beam does not change upon illumination. There is in our opinion no reason to assume that an apparatus of the first type is inferior to one of the second type; the former is, however, less complicated and less expensive.

Calibration. The calibration of the apparatus required to express deflections as changes in optical density can be carried out in two ways:

1. By measuring the deflection brought about by a known change in the measuring beam. This can be done by inserting a glass plate (1) or wire screen (see below) into the measuring beam.

2. By measuring the photocurrent produced by the measuring beam. This can be done by comparing this current by means of a "Brown converter" with a known current. Various checks on linearity of amplifier and multiplier and on influence of compensating beam are needed to make certain that the results are correct (*cf.* 3).

Apparatus using blank. An apparatus based on the same principle as a conventional spectrophotometer such as the Beckman DU could conceivably be used, provided a "blank" is used of approximately the same absorption as the cells to be measured. However, owing to technical difficulties, the sensitivity generally is not better than about 1×10^{-3} unit of optical density and may in general be insufficient for measuring spectral changes in photosynthetic tissue. Lundegardh (4) used an automatic recording apparatus of this type, but only in a narrow region where the absorption was low so that a dense suspension could be used.

Illumination of cells. Two methods of illumination for exciting the changes in absorption can be used.

1. The cells are illuminated in the vessel through which the measuring beam passes (1,2).

2. A batch of cells is illuminated outside the absorption vessel and quickly moved into this vessel to replace the nonilluminated cells. The cells are pumped around in a closed circuit, and a transparent part of the circuit at a short distance from the absorption cell is illuminated or darkened (5). In a variation of this method, the flow is stopped after the illuminated cells have passed into the absorption vessel (4).

Illumination in the absorption vessel gives the least variation in

scattering by the cells and the best time resolution. However, second-order effects on the compensation owing to scattered or fluorescent light must be eliminated. This may be done (*a*) by minimizing the scattered and fluorescent light by means of filters, (*b*) by using two sectoriated rotating disks, one in front of the phototube to cut off the light while the exciting light is being admitted through a hole in the other disk.

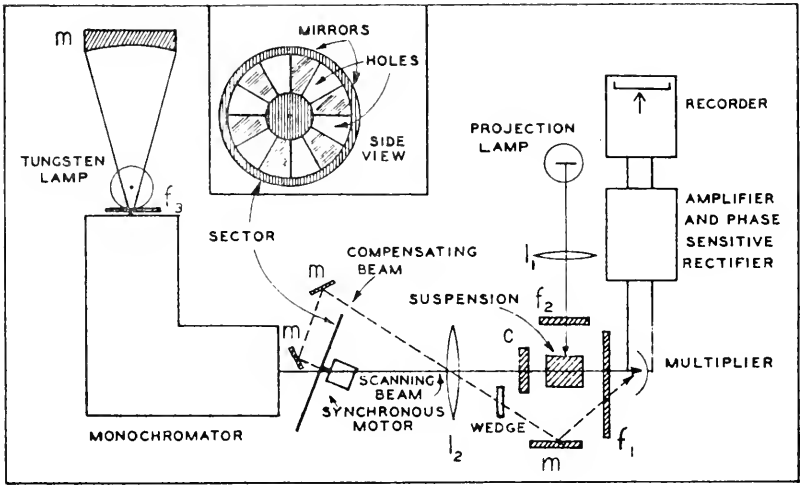

Fig. 1. Diagram of apparatus for measuring changes in absorption spectrum of a suspension of photosynthesizing cells.

Apparatus for measuring transients. Fast changes may be measured by means of a photomultiplier connected to an oscilloscope. If a measurement is completed in a short time, slow changes in intensity of the light sources, in multiplier, and in sensitivity of oscillograph amplifier may be negligible. The continuous current caused by the measuring beam may be eliminated by means of a condenser coupling or by canceling it out by a constant countercurrent.

Witt (6) succeeded in measuring in this way rapid changes in absorption brought about by light flashes. (So far, no description of his

apparatus has appeared.) Probably filters were used to prevent exciting light from reaching the multiplier.

Description of a complete apparatus. A diagram of the apparatus used by us in recent investigations (5,7,8) is shown in Fig. 1. It is a modification of one described before (1). A monochromatic beam is split into two beams by means of a rotating disc consisting of mirrors alternating with holes. The beam passing through the holes in the disc

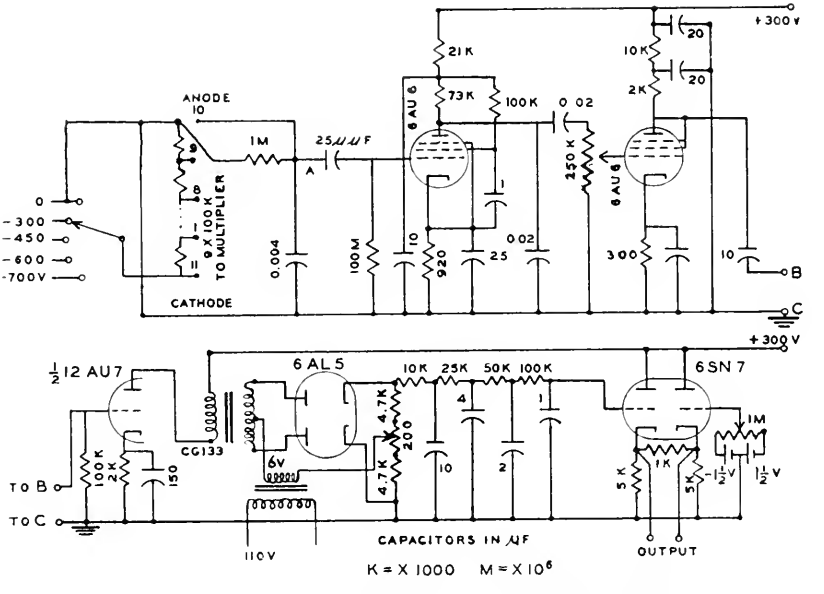

Fig. 2. Amplifier and phase- and frequency-sensitive rectifier.

is the measuring or scanning beam. This beam passes the movable wire screen *C* and the suspension. The compensating beam bypasses the suspension and causes a deflection of the recorder opposite to that of the scanning beam, keeping the indicator of the recorder on scale. The intensity of the compensating beam can be adjusted by means of a "wedge." In many experiments the intensity of the scanning beam and sensitivity setting of multiplier and amplifier was such that a

change in intensity of about 1%, brought about by moving the (calibrated) wire screen into the beam, caused a deflection of the recorder of 100 to 200 mm. This deflection was compared with the deflection caused by a change in absorption of the suspension upon illumination with a 500-watt projection lamp, the intensity of which could be varied by varying the lamp voltage.

The filters f_1 and f_2 are needed to reduce stray light from the projection lamp. In the absence of these filters, the stray light did not bring about a deflection of the recorder, since it was not interrupted with the frequency 60 c.p.s.; it was found, however, to cause a deflection when both the measuring and compensating beams were on. The filters were selected to reduce the stray light to such a low level that the projection lamp caused no deflection when a mastic suspension was used, instead of one of photosynthesizing cells.

The electronic part of the apparatus is shown in Fig. 2. The output of the three-stage amplifier is rectified by the double diode 6AL5, filtered by the four-stage filter, which removes frequencies greater than about one, and, via the balanced cathode follower, passed to a Brown recorder. The multiplier voltage and the filament voltage for the first two stages is obtained from batteries, the 6.3-volt filament voltage for the other stages is obtained from a transformer, and the plate voltage is obtained from a stabilized power supply.

TYPE OF DATA GIVEN BY MEASUREMENTS OF THE CHANGES IN ABSORPTION

Figures 3 and 4 give examples of the type of data obtained. The time course of the change in optical density in *Chlorella* appears to depend upon several factors. Figure 3 shows that the initial increase in optical density at 520 mμ upon illumination, which is caused by an unknown pigment (*cf.* 7) is much greater in an anaerobic medium than in an aerobic medium. However, upon illumination for about 1 minute, the original increase in the anaerobic medium is followed by a decrease. Figure 4 shows the time course at 420 mμ, where cytochrome *f* presumably shows a change in absorption (*cf.* 8). The changes are much larger, but less rapid, in the presence of carbon dioxide than in its absence, suggesting that carbon dioxide and cytochrome *f* compete for the same hydrogen donor.

A great number of experiments can be made. At each wavelength,

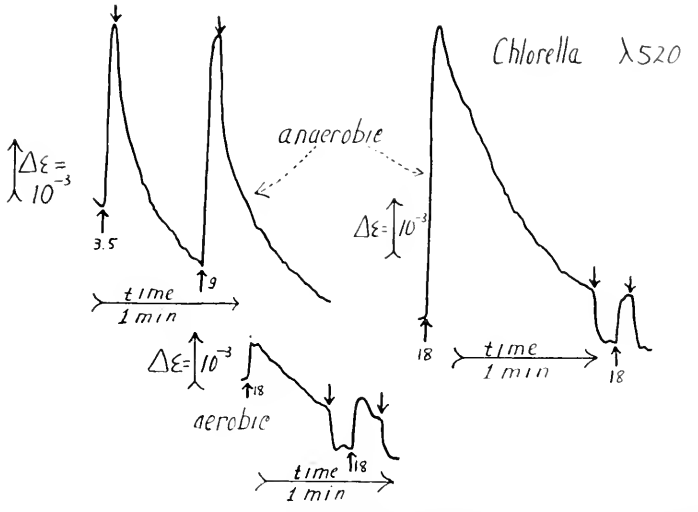

Fig. 3. Changes in optical density of *Chlorella* at 520 mμ upon onset of illumination (upward arrow) and darkening (downward arrow) as a function of time. The relative intensities of the exciting light are written near the arrows.

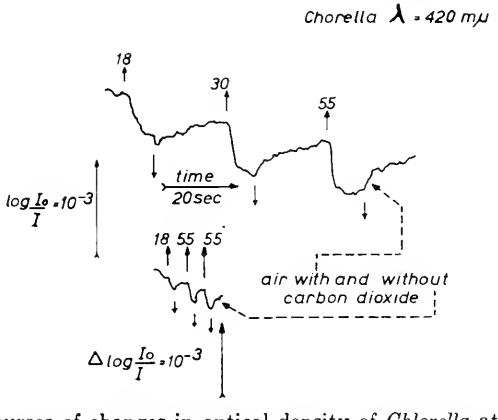

Fig. 4. Time courses of changes in optical density of *Chlorella* at 430 mμ in the presence and absence of carbon dioxide.

it is possible to measure the time course upon illumination or darkening as a function of the following parameters:

1. The composition and pH of the suspension medium. One may vary, for example, the concentration of carbon dioxide or other nutrients, oxidizing and reducing substances, poisons.

2. The pretreatment of the culture. Factors such as age, growth medium, and light intensity may be varied.

3. The intensity and wavelength of the exciting light and the lengths of light and dark periods.

Further data are obtainable by following the time course of changes in optical density caused not by light but by alterations in the medium. These measurements are not discussed in this paper.

EVALUATION OF THE DATA

Among the more useful results which can be derived from measurements of the time course at various wavelengths are the difference spectra. These spectra often allow the identification or characterization of the substances changing their absorption.

The difference spectrum can be obtained as follows. All external conditions are kept approximately constant, and a periodic sequence of light and dark periods is given. It is often found possible to select the external conditions and the patterns of illumination in such a way that at each wavelength the (change in) optical density is a periodic and reproducible function of time. A difference spectrum is then obtained by plotting the changes in optical densities which occur between two corresponding times t and t' of the time course graph as a function of the wavelength. This difference spectrum is equal to the difference of the absorption spectra of the cells at the times t and t'. It is the sum of the difference spectra of the intermediates and other substances, which change their absorption spectra. If t is the time at which the illumination period starts and t' is a time between the beginning and end of the illumination period, the difference spectra at various times t' may give a clue to the time sequence in which the intermediates change their absorption spectrum. If, e.g., one intermediate changes much more rapidly at the onset of illumination than the others, the difference spectrum obtained for a time t' shortly after the start of illumination is caused only by this intermediate.

Another family of difference spectra can be obtained selecting a different value of a parameter, such as the intensity of the exciting light or the composition of the medium. The comparison of difference spectra obtained under various conditions may greatly facilitate the analysis of these spectra in terms of the difference spectra of single intermediates. After such an analysis has been carried out, it is possible to plot the (relative) concentrations of the intermediates (or

other substances) as a function of a certain parameter, such as the time after onset of illumination or of the intensity of the exciting light. These data may be useful in the elucidation of the mechanism of photosynthesis.

Examples of the procedures outlined above can be found in references 1 and 4 to 9, and in articles in this volume.

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Reversible Bleaching of Chlorophyll *in Vivo**

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It has often been suggested (13) that in photosynthesis chlorophyll undergoes a reversible change. It could be either: (*1*) *Transformation into a "biradical," metastable state* (an electronic triplet state, with both free valencies on the same atom, or a tautomeric state, with the two valencies at different atoms); (*2*) *reduction*, either to a semiquinone or to a valence-saturated leuco compound; or (*3*) *oxidation*, also either to a radical or to a saturated product.

Transformation into the *metastable state* has been suggested as the first step in the internal conversion of excitation energy, which limits the yield of chlorophyll fluorescence to 25% (10) or 33% (7), *in vitro*, and 2% to 3% *in vivo* (7). According to Franck (5), photosynthesis probably occurs by reactions of metastable chlorophyll *a* molecules. According to Livingston and Ryan (12), these molecules are co-responsible for changes in the absorption spectrum of illuminated chlorophyll solutions in the photostationary state; Livingston and Ryan (12) and Livingston, Porter, and Windsor (11), using condenser flashes with synchronized absorption measurements, found that during an intense flash, up to 90% of chlorophyll (in a $10^{-6}M$ solution) can be present in the metastable state. Livingston and Ryan's (12) steady-state experiments indicated bleaching at 403 mμ and enhanced absorption at 439.5 to 524.5 mμ; whereas their flash results showed bleaching at 468, 470.5, and 477.5 mμ, and enhancement of color only at 524.5 mμ. However, according to the newer flash data of Livingston, Porter, and Windsor (11), analyzed by Livingston (9), enhancement extends over the range 450 to 560 mμ, with a sharp peak at 475 mμ and a shoulder at 520 mμ.

Evstigneev and Gavrilova (4) found that reduced chlorophyll *a*, obtained by illumination of phenylhydrazine-containing solution in

* This work was carried out with the assistance of the Office of Naval Research.

toluene, has absorption bands at 518 mμ and 585 mμ. Both bands were attributed to a semiquinone: the 518-mμ band to its ion and the 585-mμ band to the nondissociated form.* Krasnovskii (6) has suggested that chlorophyll participates in photosynthesis by reversible reduction to the semiquinone state.

Studies of reversible photobleaching of chlorophyll in O_2-free methanol (9,10,14) and of its reversible photooxidation by Fe^{+++} in methanol (15) and by quinone in rigid solvents (8) revealed an enhanced absorption in the region 450 to 530 mμ, but no sharp bands were detected. The brown intermediate in the phase test (probably, an ionized enol form) of chlorophyll *a* shows a strong band at 524 mμ, with a shoulder at 486 mμ, and weaker bands at 645 and 683 mμ (16).

It thus seems that, *in vitro*, reduced chlorophyll *a* is characterized by bands at 525 mμ and 585 mμ, metastable chlorophyll *a* by a band at 475 mμ, and ionized chlorophyll by bands at 486 mμ and 524 mμ. Reversible oxidation increases absorption in the same general region, apparently without producing a sharp new band. The absorption in the red decreases in every case.

Duysens (2) (and also Witt (17)) noted that illuminated *Chlorella* cells show, in addition to spectral changes attributable to the oxidation of a cytochrome (and perhaps also to the reduction of a pyridine nucleotide) (1), a sharp new absorption band at 515 mμ and a somewhat smaller "negative" band (i.e., selective decrease of absorption) at 478 mμ. Duysens attributed the two changes to the transformation of an unidentified pigment, whose "dark" form absorbs at 478 mμ and whose "phototropic" form absorbs at 515 mμ. Witt noted the 515-mμ band also in plants exposed to an intense light flash. Duysens observed no change in the absorption in the red region, thus apparently precluding the attribution of the effect at 515 mμ to chlorophyll.

Using an apparatus similar in principle to that of Duysens (3) but somewhat different in construction, we have been able to observe a decrease in absorption of illuminated *Chlorella* cells in the red. In our apparatus, the modulated photomultiplier output was amplified through three sharply tuned and six narrow-band staggered stages; by means of a phase-inverting parallel twin-T tuned network, a considerable portion of the signal was negatively fed back from the fourth stage to the input. The ultrasharp tuning and increased feed-

* Linschitz and co-workers (8A) found no evidence of the presence of free radicals in this system by the method of paramagnetic resonance.

back were necessitated by difficulty of discriminating between fluctuations in the fluorescence excited by the (very intense) actinic light and changes in the (much weaker) measuring light. After the ninth stage of amplification, the signal was rectified, compared, and, by means of a balanced-plate cathode-follower, fed into a Brown recorder (as in Duysens' instrument).

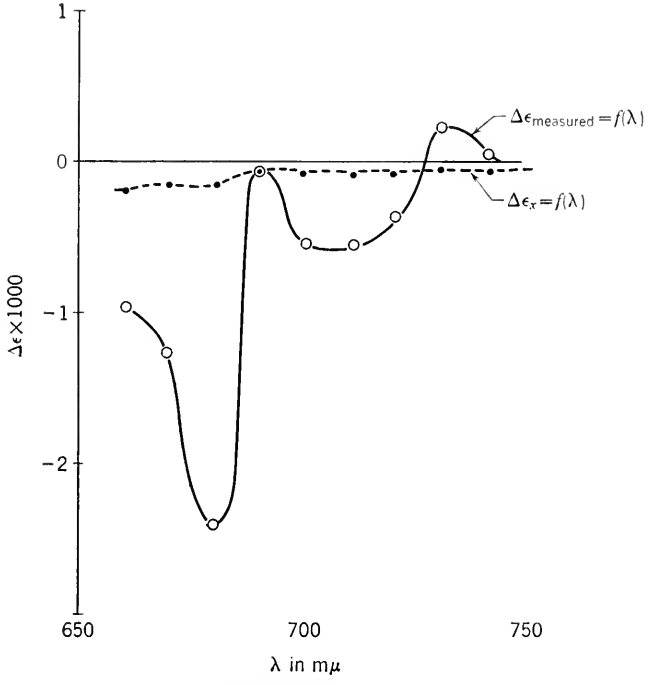

Fig. 1. Reversible changes in *Chlorella* spectrum during illumination. Dashed line: estimated correction for fluorescence.

Chlorella cells, grown in our laboratory, were washed, suspended in carbonate, and refrigerated until needed. The cells were used as taken from the refrigerator (optical density of suspensions, 0.45, at 680 mμ, corrected for scattering). The actinic light was furnished by a tungsten lamp (1000 watt, GE 1000T20, 120 volts); the entire side of the cuvette was uniformly illuminated. Before using a sample for systematic measurement, a check was made at several selected wave-

lengths to see whether the cells showed the normal response to illumination.

The apparatus reproduced with excellent agreement the earlier work of Duysens; in addition, it clearly showed absorption changes in the red.

A typical differential absorption spectrum is shown by solid line in Fig. 1. At 680 mμ, the optical density of illuminated cells can be 0.25% lower. Exact comparison of this decrease with the increase at 515 mμ is not possible because we had to use different exciting lights in the two regions. However, since the two effects are of the same order of magnitude, the assumption is permitted that they are both caused by a reversible change in chlorophyll *a*. Spectroscopically, this change is most similar to that observed by Krasnovskii *et al.* (and by Evstigneev and Gavrilova) upon reversible reduction of chlorophyll *a in vitro*. The smaller changes farther in the red (decline of absorption at 710 to 715 mμ, and increase at 730 mμ), as well as the bleaching at 478 mμ (already noted by Duysens), remain to be interpreted. Several reversible changes of chlorophyll may occur at once in the cell, e.g., the formation of metastable triplet molecules may be superimposed on that of the semiquinone. It will be noted, however, that the effect observed *in vivo* at 475 mμ is opposite in sign to that expected from the formation of metastable chlorophyll *a*.

Discussion

Duysens: I, too, made some experiments in red while I was working in your laboratory. I used illumination of presumably lower intensity than you did—the same illumination at 515 and 680 mμ. It was rather difficult to exclude completely any change at 680 mμ, but I think the changes there were less than one-fourth of those at 515 mμ. So it is possible that the changes which you find occur only at higher light intensity and are not correlated with those at 515 mμ.

Rabinowitch: That is a possibility, and I would not say that there is here a direct experimental disagreement of the kind we are only too familiar with in photosynthesis. However, according to Coleman and Holt, the effects are of the same order of magnitude in the green and in the red; but, since two different kinds of illuminating light were used in these two regions, we as yet cannot compare them exactly.

Strehler: I would like to make two points: One, that using Dr. French's apparatus we were not able to find any changes on the red side of about 670 mμ, while we did get large changes around 648 mμ. Second, apparent changes in transmission could be due to fluorescence. How can you rule out the possibility that

changes in fluorescence yield may occur when you add the cross illumination? Fluorescence caused by the measuring light is modulated, and any fluctuation in it will be picked up by your phase-sensitive detector. If the sense of the change is proper, you will get in this way something that could be interpreted as a decrease in transmission.

Rabinowitch: Unless the actinic light caused changes in the spectral composition of fluorescence and not only in its intensity, how could you get in this way both positive and negative effects? However, the commonly made assumption that the spectrum of fluorescence does not change when changes in fluorescence intensity are observed *in vivo* is in need of experimental confirmation. If this happens, the meaning of many data in the literature becomes questionable.

Strehler: You are passing through an absorption band here (660 to 720 mμ) going from wavelengths that do excite chlorophyll fluorescence into a spectral region where chlorophyll does not absorb. I am not saying that your effect in the red is necessarily due to this, but this possibility worried us when we measured our 648-mμ band. I believe we can rule it out in our case, because we put a red filter in front of the photomultiplier, which transmitted only the fluorescence, and with this filter in place we didn't observe any significant signals upon cross illuminating.

Rabinowitch: One probably has to consider this point more carefully than we did, but it still seems to me that you cannot get in this way both positive and negative effects.

Chance: This difficulty would be minimized in the double-beam apparatus we use. If you could change your apparatus correspondingly, you may get an adequate control.

Strehler: Provided the excitation of fluorescence by the two different wavelengths is the same. Actually, by placing another monochromator after your absorption cell, you can remove most of the fluorescence, since it forms a broad band.

Chance: Yes, but you may be unable to get enough light into the analyzing monochromator.

Rabinowitch: Of course, when he measured at 515 mμ, Holt did use a filter to cut off fluorescence.

Strehler: The changes at 515 mμ cannot be due to fluorescence.

Rabinowitch: When we measured at 515 mμ, the fluorescence-removing filter did not change the results. This means that at 515 mμ, the effect of modulated fluorescence was insignificant compared to the absorption effect. If the total observed effect were much stronger at 515 mμ than at 680 mμ, one could suggest that fluorescence was much more significant in the latter region; but, as I said before, Coleman's impression is that the effects in the two regions are about the same; and, if this is so, one can infer that the role of modulated fluorescence is insignificant in the red, too.

Linschitz: If we accept your data at their face value, there would still be a problem of showing that the changes at 680 and 515 mμ are correlated. We have found that, *in vitro*, these two bands may change at different rates. There are probably two different products which cause the change.

(Remarks added in manuscript)

Coleman: The possible influence of changes in modulated fluorescence, pointed out by Dr. Strehler, was taken into account by Dr. Holt and myself, although we did not include the discussion of this point in our report. Since it has been raised by Dr. Strehler, here is a brief summary of the reasons why we considered this influence negligible.

In contrast to the constant actinic light, the modulated scanning light does produce a 60-cycle modulated fluorescence to which the photomultiplier can respond. In the apparatus as we used it, this modulated fluorescence is compensated when the actinic light is off ("darkness"); but, from general knowledge of the fluorescence phenomena *in vivo*, we must consider it possible—even likely—that switching on the actinic light—changing from "darkness" to "light"—will change the intrinsic capacity of the cells to fluoresce and thus affect (probably, increase) the intensity of the modulated fluorescence. The apparatus will react to such a change in modulated fluorescence as if it were a change (decrease) in absorption in the fluorescence vessel. We thus have:

$$\Delta A \text{ (true)} = \Delta A \text{ (measured)} - \Delta A_x$$

where ΔA_x is the change in the modulated fluorescence reaching the detector, caused by switching on the actinic light.

To determine the order of magnitude of ΔA_x, the scanning beam was rerouted, so that it traversed the suspension orthogonally to its usual path. In this way, only a small scattered fraction of the exciting light entered the photomultiplier, instead of the full transmitted beam, as in the usual arrangement. Fluorescence, on the other hand, was collected from the same volume, subtending the same angle at the photomultiplier.

First, heat-killed cells were illuminated with the rerouted beam; the scattered scanning light was compensated, and the deflection d_1 (corresponding to 1% change in the beam intensity) was recorded, as usual, with the help of a calibrated neutral filter. Although d_1 changed slightly when the actinic light was turned on (probably because of scattered actinic light modulated by line ripple voltage), this change was negligible. The suspension of dead cells was then replaced by one of live cells, having the same optical density, and the procedure was repeated, giving the deflections d_2 in darkness and d_3 when exposed to actinic light. We can expect d_2 to be the same as d_1, and this was in fact the case within a few per cent. The value of ΔA_x, the increase in fluorescence caused by the actinic light, was calculated from the equation

$$\Delta A_x = \frac{K\,[2d_3 - (d_1 + d_2)]}{d_1 + d_2}$$

where K is a correction factor ($\simeq$1.4) for the loss of intensity of scanning beam caused by additional reflections required for its rerouting.

The resulting correction curve, $\Delta A_x = f(\lambda)$, is shown by the dashed line in Fig. 1. At λ = 680 mλ, ΔA_x contributes about 7% of ΔA (measured). Where ΔA (measured) is smaller, ΔA_x contributes proportionally more, but in no case is the error large enough to alter the shape of the ΔA-curve.

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Reaction Patterns in the Primary Process of Photosynthesis

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In the primary process of photosynthesis light is absorbed indirectly or directly by chlorophyll. During this process the chlorophyll or chlorophyll complex changes into an unknown excited state. With the aid of the energy of this state water is split in an unknown way into oxygen and hydrogen (1). In a secondary process this hydrogen reduces carbon dioxide to carbohydrates. You know of the great success in the investigation of this carbon dioxide cycle here in America (2). But there is little knowledge about the first step which transforms light into free chemical energy. A special state of chlorophyll during photosynthesis has not yet been observed. Furthermore we cannot tell anything about the mechanism of hydrogen production because the usual methods, for instance, the measurement of oxygen production, are not sufficient to detect the fast reaction in the primary process.

We have tried to measure reaction patterns in the primary process by looking for *fast changes of absorption* immediately after flashes of light (3).

These changes of absorption cannot be detected by the usual type of absorption measurements because the reactions are very fast and the changes very small. There are, furthermore, technical complications due to the scattering of the incident light by the plants. Therefore we have developed a sensitive apparatus by which we could measure absorption changes in times between $\sim 10^{-5}$ second and several seconds.*

The experiments were made with various leaves, *Chlorella,* and chloroplasts. The photosynthesis was induced by periodic flashes of light. The spectrum of the flash lay between 620 mμ and 700 mμ.

* Changes of the absorption in steady light were reported in connection with redox reactions of cytochromes and pyridine nucleotides (4).

We were searching for changes of absorption between 400 mμ and 580 mμ.

When photosynthesis is started by flashes of light, there is a rapid increase of a new absorption band at ~515 mμ. At the same time (within the accuracy of measurement) a decrease of absorption takes

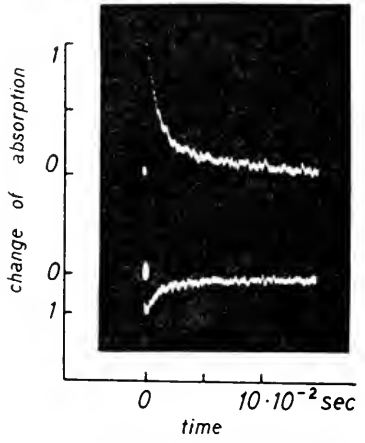

Fig. 1. Change of absorption of *Chlorella* as function of time. At time $t = 0$ the *Chlorella* were lighted by a flash of light, $t_l = 5 \times 10^{-3}$ second, $t_d = 0.5$ second. Temperature 19°C. Upper curve: change of absorption at 515 mμ. The change from 0 to 1 corresponds to an increase of absorption. Lower curve: change of absorption at 475 mμ. The change from 0 to 1 corresponds to a decrease of absorption.

place with a maximum at about 475 mμ (3) (Fig. 1).* The relative change of absorption is only of the order of a tenth of a per cent. Using flashes as short as 3×10^{-5} second we observe a change of absorption within the duration of the flash. This change must, therefore, take place within the time of 3×10^{-5} second or less. In the dark time after the flash, the change of absorption disappears completely within ~10^{-2} second.

With an increase of *light intensity* there is also an increase of the change of absorption (Fig. 2). At high intensities there is a saturation of the absorption changes. The intensity at the saturation point is of the order of that which suffices to saturate photosynthesis.

* In text of all figures t_l means: duration of light flash, t_d: dark time between the flashes.

At *temperatures* near 50°C. the change of absorption decreases with increasing temperature (Fig. 3). At this temperature photosynthesis also declines. With decreasing temperature we can observe by using short saturating flashes that at 515 mμ the fast increase of absorption is independent of temperature (Fig. 4). But in the dark time after the flash the decline of the change of absorption is a function of temperature. Between 30°C. and 5°C. we measured half-times of

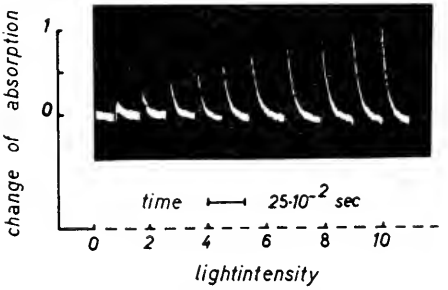

Fig. 2. Change of absorption of *Chlorella* at 515 mμ as a function of flash light intensity, $t_l = 5 \times 10^{-3}$ second, $t_d = 0.24$ second. Temperature 18°C. The abscissa (broken line) belongs to a curve that connects the maxima of the changes of absorption.

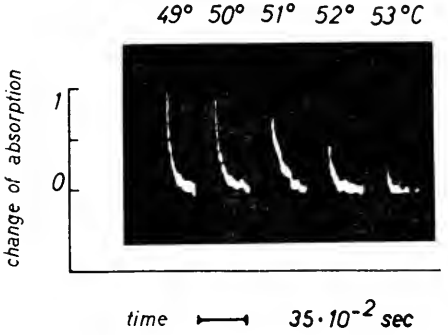

Fig. 3. Change of absorption of *Chlorella* at 515 mμ as a function of time at different temperatures around 50°C. $t_l = 1 \times 10^{-4}$ second, $t_d = 3$ seconds.

1×10^{-2} second and 7×10^{-2} second (see arrows in Fig. 4). Emerson and Arnold (5) found a reaction period of similar length indirectly by measurements of oxygen production in photosynthesis. This reaction period of photosynthesis is perhaps identical with the lifetime of the changes of absorption.

These experiments make it probable that the changes of absorption are a direct reflection of partial processes in photosynthesis. That the last change is one of the first steps in the primary process can also be shown in the following way.

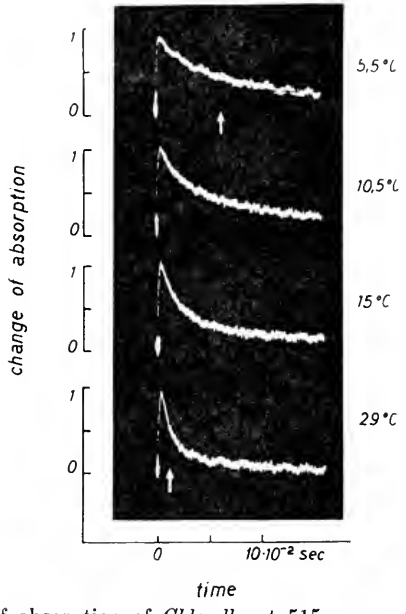

Fig. 4. Change of absorption of *Chlorella* at 515 mμ as function of time at temperatures between 5.5°C. and 29°C. At the time $t = 0$ the *Chlorella* were lighted by a flash of light, $t_l = 4 \times 10^{-3}$ second, $t_d = 0.76$ second. The arrows are indicating the half-time of the lifetime.

It is known that HCN (6) and quinone act as *inhibitors* by blocking the cycle of carbon dioxide. But the change of absorption takes place even in the presence of these poisons. Furthermore one can see the change of absorption of chloroplasts outside the living cell (3). On the other hand, urethane is a strong poison for the primary process. Correspondingly we found with increasing urethane concentration a decrease in the change of absorption. This inhibition is reversible; after washing you can restore the original change.

To obtain more accurate information about the mechanism of this process we measured the change of absorption as a function of several parameters.

By varying the *duration of the flashes*, we obtained the different shapes of absorption changes with respect to time, as shown in Fig. 5. In these experiments we always used saturating intensities. Passing from short to longer flashes there is an increase in the change of absorption. At a critical flash time this increase in the change of absorption stops, and a second strong but slow increase of absorption follows. This shows the existence of two phases of the change of absorption: a first fast one and a second slow one.*

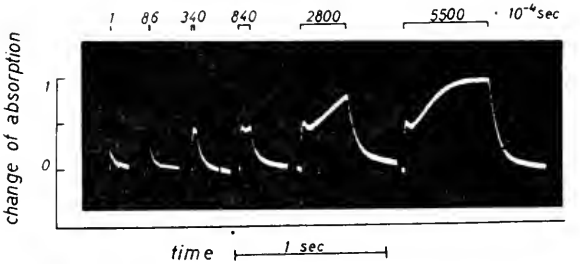

Fig. 5. Change of absorption of *Chlorella* at 515 mμ as function of time by lighting the *Chlorella* with flashes of light of different durations (see figures about the picture). $t_d \approx 1$ second.

The first fast phase of the absorption changes is as independent of temperature as the effect of short flashes (Fig. 6). By contrast the second phase decreases with decreasing temperature until at 5°C. there is almost no second phase. In the dark after long flashes the decline is again a function of temperature, as we have seen after short flashes.

The second slow phase depends also on the *dark time between the flashes.*

A second phase can be observed also in the decrease in absorption at 475 mμ. The change of this phase by different temperatures and dark times is very similar to the change of the second phase at 515 mμ. This means that the change of absorption at 515 mμ and 475 mμ probably belongs to one and the same reaction.

On the basis of these experiments we postulate three reactions during the primary process.

* Duysens has reported changes of absorption in steady light at 515 mμ and 478 mμ (4). Probably these changes correspond to the end point of the second slow phase.

1. The first rise of absorption is probably due to a photochemical reaction F. In this reaction unknown molecules X (absorption band ~475 mμ) are transformed to Y (absorption band ~515 mμ).

2. During the temperature-dependent, slow second rise of absorption a rate determining chemical reaction C must be involved. This reaction releases further molecules X, which are also transformed by light to Y.

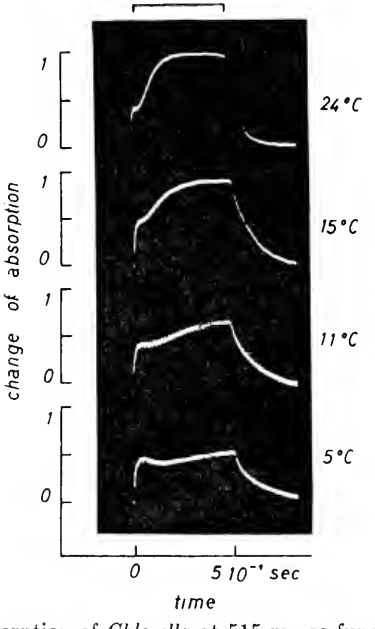

Fig. 6. Change of absorption of *Chlorella* at 515 mμ as function of time at temperatures between 24°C. and 5°C. t_l = 0.52 second, t_d = 0.93 second.

3. The temperature-dependent decline in the dark time after the flash is connected with a chemical dark reaction D. In this reaction the molecules Y built up in the light, are reacting with one or more unknown molecules Z to form X and Z'.

After the discovery of *chlorophyll fluorescence* in plants (7) the change of fluorescence was used for investigations of photosynthesis. Unfortunately there exists no definite correlation between the change of fluorescence and the occurrence of other reactions on chlorophyll for large molecules. This fact makes it difficult to interpret the meas-

urements of fluorescence. Therefore it is interesting to make a comparison of changes in absorption and fluorescence. We made such measurements on the same object (Fig. 7). The plot of both with respect to time shows conformities as if both changes belong to reactions with the same substance. Because the change of fluorescence belongs to chlorophyll, it is likely that the change of absorption also belongs to chlorophyll or to a substance which reacts directly with

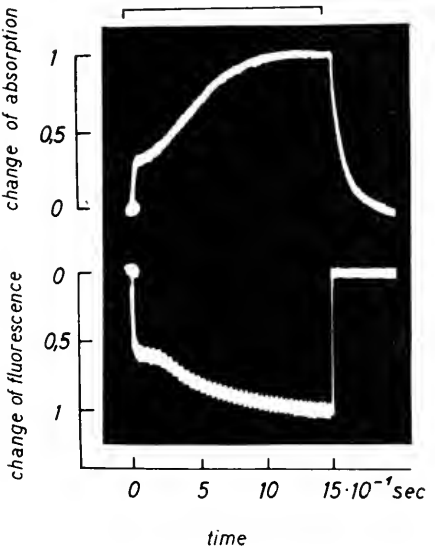

Fig. 7. First picture: Change of absorption of *Chlorella* at 515 mμ as function of time. Second picture: Change of fluorescence of *Chlorella* at 700 mμ as function of time. $-t_l = 1.5$ second, $t_d = 0.8$ second.

chlorophyll. If so, the change of absorption at 475 mμ and 515 mμ characterizing the unknown substances X and Y must be attributed to a chlorophyll complex or to a substance directly reacting with chlorophyll.*

These changes of absorption at 515 mμ and 475 mμ are found also in chloroplasts separate from the living cell. It is of interest that the decline of the change of absorption in the dark time after the flash is nearly the same, whether there is a Hill reaction (with quinone or

* The typical change of fluorescence was described 12 years ago by H. Kautsky and U. Franck on *Ulva lactūca* (*Biochem. Z.*, *315*, 156 (1943)).

ferricyanide) going on or not (Fig. 8*a* and *b*). But using phenol-indophenol and other dyes as a Hill oxidant the decline is strongly dependent on the concentration of this substance. At concentrations of $\sim 10^{-4}$ mole per liter it is possible to shorten the lifetime of the change of absorption (at constant temperature) by a factor of about ten. The effect is reversible. One explanation for this effect may be the following: the unknown substance Z is replaced by phenol-indophenol, which reacts directly with Y. Because phenol-indophenol is reduced,

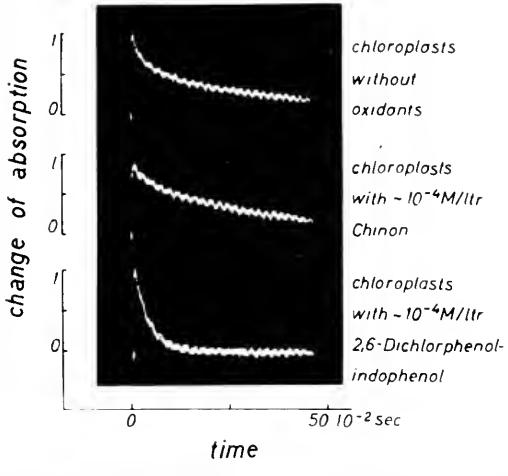

Fig. 8. Change of absorption of chloroplasts (spinach, pH = 6.5) as function of time under different conditions. $t_l = 10^{-2}$ second, $t_d = 1.0$ second. Temperature 13.5°C.

Y can be a reduced substance and X the oxidized form. We hope to learn more about the primary process by exploring this possibility of interfering with the reaction system in chloroplasts.

The results of these experiments are of a preliminary character. After further experiments we shall make a detailed comparison of these effects with the results of other authors. I hope to have demonstrated that, by means of the method which was used in all these experiments, it is possible to measure reaction patterns of the primary process from $\sim 10^{-5}$ second up to the time required for the reduction phase of the Hill reaction.

Discussion

Rabinowitch: Dr. Witt, may I ask you, can you describe in a few words the kind of flashes you used?

Witt: We used a spinning wheel for the long flashes, and for the short flashes we used electrical discharge tubes.

Rabinowitch: For the longer flashes you simply used rotating discs in front of what kind of light?

Witt: In front of a tungsten light.

Arnold: What was the position of the cells?

Witt: Above the multiplier to catch all scattered light.

Rabinowitch: How often did you flash this lamp?

Witt: Up to 200 cycles a second.

Rabinowitch: And it gave enough light to saturate?

Witt: Yes. Only with 3.10^{-5} second flashes did we observe a loss of the saturation approximating 10%.

Chance: Were you measuring at wavelengths other than 515?

Witt: At 475; and not precisely at 420 and 555. We intend first to get information about the change of absorption at 515 and 475 as a function of several parameters. Then we intend to go on to other wavelengths.

Chance: But these fast and slow effects might be rather different at other wavelengths.

Witt: Yes, I think so, and I hope to see the differences.

Wassink: Have you ever extended the light period longer than what you showed—to more than 1 second?

Witt: Yes, we have seen marked changes of absorption from between 1 second and 1 minute, after longer dark periods.

Wassink: From what I remember of our fluorescent measurements they extend certainly much longer than something of the order of a few seconds. They extend up to the order of a minute.

French: Dr. Strehler will have some absorption curves for longer times.

Wassink: Is that a definite end-phase you reach, or is something going on after that?

Witt: We measured all effects in the steady state of periodic flashing light, and if you use long "flashes" you can see more—but slower—changes of absorption with an end-phase after some minutes.

Lumry: Just one observation. This is intriguing but certainly not a fact yet. Those of us who have been working on quantitative Hill reaction characteristics are concerned because we did not check the early work of Holt and French who used 2,6-dichlorophenol indophenol as their oxidant. Also we did not check the recent work of Tom Hill working in R. Hill's laboratory, and he uses this oxidant, too. We have studied a lot of things with hydrogen ion evolution and oxygen evolution in the reducing of the Hill oxidant. We find good agreement in these reactions, but there are drastic differences between our results and those apparently observed with the 2,6-dichlorophenol indophenol. So maybe the effect that you have been showing here has been showing up all the time and we have not grasped it until recently.

French: Hill has been using purified dye which makes the difference.

Rabinowitch: It is strange that there should be a difference. After all, this is just a different quinone.

Rosenberg: In the experiments with the very short flashes your photographs of the stationary diagrams on the oscilloscope represented many flashes. Did you ever measure what happens with the first flash? I am wondering whether the absorption changes take some time to appear and whether you need several flashes to produce a steady state.

Witt: If you measure the first flash, i.e., after a long dark time, the increase of absorption and the subsequent decline during the first dark period is greater than after many periods of flashing light.

Gaffron: Quinone, a strong oxidant, quenches the fluorescence in algae. I suspect that, if the absorption changes found with 2,6-dichlorophenolindophenol are really due to changes in chlorophyll, then one should observe a quenching of the fluorescence by phenolindophenol, comparable to that produced by quinone.

Duysens: I don't think the change at 520 mμ represents, in *Chlorella*, a change in the chlorophyll *a* molecule because you would expect a decrease of the blue absorption band and also a change in the red absorption band. However, the change at 680 mμ I found was appreciably smaller—if it exists at all—than those at 520 and 475 mμ.

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Spectroscopy of Flash-Illuminated Chloroplasts*

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The existence of an excited metastable electronic state of chlorophyll has been postulated for some time as a crucial intermediate in chlorophyll photosensitized reactions (3). Porter and co-workers have recently shown that many organic substances can be converted with high yield to their metastable states when excited with the intense light of a flash. Direct proof of the existence of such a state can be obtained by a rapid recording of the absorption spectrum immediately after the flash (6). Livingston (5) and Linschitz (4), using these methods, succeeded in measuring the absorption spectrum of the lowest metastable state of chlorophyll dissolved in oxygen-free organic solvents. In this paper we are reporting results of our attempts to observe the same phenomenon in flash-illuminated chloroplasts.

The flash and spectrographic equipment were those of Livingston.† In each experiment two flashes of light were used, separated in time by about 30 to 40 microseconds. The first was the photolytic flash, about 10 to 20 microseconds half-life, which concentrated light from a 50-joule gas discharge upon the sample. The second was a spectroscopic flash, which sent a collimated beam through the sample and then into the spectrograph. The entire absorption spectrum from 440 to 680 mμ was recorded instantaneously on a spectrographic plate. Scattering of some of the photolytic light by the sample into the spectrograph was restricted to the long-wavelength region by the use of a red cellophane filter around the sample tube. The filter reduced the amount of energy available at the sample, but the red light transmitted was intense enough to convert into its metastable state over 70% of the chlorophyll in a benzene solution which was used as a control.

* These studies were aided by contract between the Office of Naval Research, Department of the Navy, and the University of Minnesota, NR 120-306.

† We are indebted to Dr. Livingston for permission to use these facilities. We also wish to acknowledge the generous assistance of Dr. David Tanner, who patiently instructed us in the operation of the equipment.

The chloroplasts were prepared from sugar-beet leaves, washed at least three times, suspended in buffer without added oxidant, and equilibrated with atmospheres of either air, nitrogen, or water vapor only. All measurements were made below 20°C.

We had expected to find the same short-lived substance which Witt found after illumination at lower intensities (7). In fact, we had thought that this substance might be present in much larger amounts than in his experiments because of the great intensity of our flashes and because of the short time elapsing between excitation and analysis. Accordingly, we had anticipated that we would be able to determine the entire visible absorption spectrum of Witt's intermediate. On the contrary, our experiments were all negative. That is, we could observe no change in absorption spectrum as a result of the flash, within the experimental limit of about 5% in the optical density. Negative results were also obtained in a few experiments with *Chlorella* suspensions. To justify the difference in behavior of our suspensions with that of chlorophyll in organic solutions, the following possibilities must be considered.

1. Chlorophyll molecules *in vivo* are all equivalent but are so altered that the lifetime of a molecule in the metastable state is shorter than that in dilute organic solutions. A shorter *in vivo* lifetime would be expected as a result of increased concentration alone if, as suggested by Livingston (5), the metastable state decays by a bimolecular reaction involving a second excited or unexcited pigment molecule. Such a reaction, however, must be prohibited in the plant since it would not be photochemically productive. Thus the decrease in metastable state lifetime must be due to an accelerated, specifically *in vivo* reaction leading to useful photoproducts either through the metastable state or through a new state characteristic of chlorophyll plus an additional reactant of photosynthesis. The half-time of such a reaction would have to be less than 10 microseconds. Perhaps this short a reaction time should not be surprising since the primary photochemical step in photosynthesis must compete very favorably with the known rapid and probably wasteful reaction of oxygen with chlorophyll's metastable state. Since the latter reaction can proceed with zero energy of activation, but apparently does not occur to an appreciable extent even at the relatively high oxygen concentration present in air-saturated supensions of healthy cells, the reaction of the metastable state with the substrate of photosynthesis must indeed be

a very rapid one. This substrate, it should be emphasized, is not a very diffusible substance since it is present in well-washed chloroplast preparations. Perhaps it is some portion of a protein or a carotenoid molecule bound closely to chlorophyll.

2. Another possibility which seems to be equally consistent with our results is that only few chlorophyll molecules, or perhaps none, ever have an opportunity to undergo internal conversion into the metastable state. The electronic excitation energy is transferred by induction resonance to a small population of distinctly different pigment molecules, which may be some pigment different from chlorophyll or chlorophyll molecules so situated as to be able to act as efficient traps for electronic quanta. We may imagine the mechanism of these trapping centers to function along lines proposed by Franck and Livingston (8) for self-quenching among dye molecules. The electronic energy passes undegraded to these centers, which will efficiently depopulate the excited singlet state of chlorophyll because they are associated with a substance which will induce internal conversion out of the singlet state. The latter induction reaction occurs because the centers are a different kind of dye molecule with fluorescence emission farther to the red than chlorophyll, or because they have extreme configurational distortion. A trapping center of a different type than chlorophyll *a* is possible providing its concentration is truly so low as to be undetectable in the careful studies which have been made of absorption and emission spectra in plants. Franck has recently proposed that these trapping centers may be chlorophyll in the metastable state and we cannot exclude such an interesting possibility completely (2). We feel, however, that sufficient chlorophyll molecules should have remained after the flash in our experiments if all chlorophyll molecules have an equal chance of becoming trapping centers by reason of an equal probability of passing into the metastable state. Thus it seems necessary again to suppose that only a small proportion of all chlorophyll molecules are properly situated to pass rapidly into the metastable state. It may not be wise to maintain a preference in this matter at the present time, but we believe the most attractive picture to consist of a photosynthetic unit established purely by geometry about certain chlorophyll molecules closely associated with subsidiary reactants of photosynthesis and electronically altered by them in such a way as to enhance the rate of internal conversion out of the excited singlet state into a state which

can react immediately with the second reactant. Electronic quanta travel by induction resonance to these trapping centers where they are rapidly and efficiently converted into chemical energy. The small population of these centers in itself allows only a very small instantaneous concentration of pigment molecules in the latter state and thus explains our negative findings.

Witt's observation (7) that the intermediate he studied was limited by a saturation process may provide some support for the latter picture; but we do not yet know that Witt's spectral changes are due to the trapping center. They may be due to some pigment, perhaps one of the cytochromes as Duysens has suggested (1), well removed in the reaction chain from chlorophyll and other molecules immediately involved with the pick-up and conversion of excitation energy into chemical energy.

Discussion

Frank Allen: Were your samples in complete darkness before the bright flash?

Rosenberg: We tried complete darkness for up to a half hour and some times dim light—room light—and we varied the conditions in the presence or absence of oxygen or the presence or absence of a supplied oxidizing agent and we still could observe nothing.

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Absorption Spectrum Changes in *Chlorella* and the Primary Process

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The primary chemical reaction in photosynthesis is generally believed to lead to intermediates (both reducing compounds and precursors of molecular oxygen) which are capable of reacting back to form excited chlorophyll. This reaction is presumably responsible for the weak chemiluminescence emitted by photosynthesizing tissues following their illumination (1).

If the primary oxidants and reductants in photosynthesis differ from their dark precursors in color, it should be possible experimentally to learn something of their nature through sensitive spectrophotometric measurements. The technique of measuring biochemical kinetics through absorption spectrometry has been developed to a high degree by Chance (2) and in a parallel manner by Duysens (3), Lundegårdh (4), Witt (5), and French (6), each worker using somewhat different techniques.

During the summer of 1955, through the courtesy of Dr. C. S. French, it became possible for us to use his sensitive derivative spectrophotometer to examine absorption spectrum changes in *Chlorella* induced by illumination. Some years earlier, similar experiments were attempted by the senior author at Oak Ridge. At that time changes in transmission in the region 500 to 550 mμ were found, but since we were looking for cytochrome bands at the time, and since the spectral difference bands were quite broad, we rejected these early results as probably due to some instrumental error or scattering change.

Duysens several years ago described striking absorption changes in *Chlorella* during illumination. The present studies confirm Duysens' earlier work and in addition uncover a number of new difference spectrum bands hitherto unreported, as well as some unexpected kinetic behavior and a probable relationship of the spectral changes to the process of photosynthetic luminescence. These studies show that the

magnitude and even the sign of the spectral changes as a result of illumination are dependent on the manner, intensity, and duration of exposure to light and on the time elapsing between illumination and measurement.

MATERIALS AND METHODS

The details of the experimental methods and modifications of the derivative spectrophotometer used in these studies are described elsewhere (7). *Chlorella pyrenoidosa* was cultured under incandescent light at *ca.* 20°C. in *ca.* 5% CO_2-air.

Three methods of illumination were used in these experiments:

1. Direct cross illumination *during* measurement. Filters were used to protect the phototube from the photosynthesis-inducing light and to pass the measuring beam.

2. A circulating system in which the algae were illuminated for periods of 5 to 60 seconds outside of the spectrophotometer and then pumped rapidly through a cuvette placed in the measuring beam.

3. Same as the second method, except that the cells were illuminated for a fraction of a second in the flow system prior to measurement.

In order to compare the luminescence of *Chlorella* under various conditions with the simultaneous absorption spectrum changes, the flow of algae from the illumination chamber (in the second and third methods above) was divided into two parallel streams, one passing into the difference spectrophotometer, the other in front of a photomultiplier sensitive to the red luminescence emitted by the plants. The respective signals were recorded simultaneously on Brown Electronik recorders.

RESULTS

1. In conformity with Duysen's findings, we observed spectral changes in the wavelength regions 480 mμ and 520 mμ. We did not, however, observe the band around 420 mμ which Duysens noted, either because our apparatus may have been less sensitive than his or because our algae were grown under different conditions.

2. We observed striking induction changes not mentioned by Duysens. These effects are illustrated in Fig. 1. In Fig. 2 are illustrated the changes in absorption as a function of incident light in-

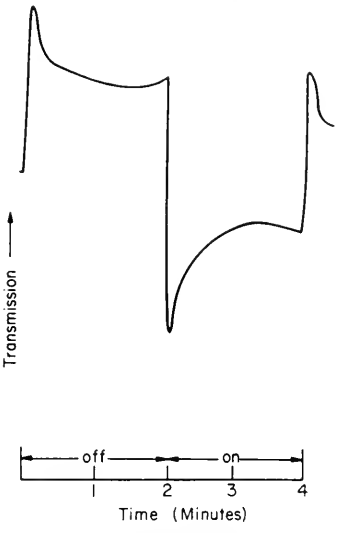


Fig. 1. Time course of transmission of *Chlorella* at 525 mμ during direct illumination. O.D. density about 0.8; light intensity (red) about 400 foot-candles. Temperature, 25°C.

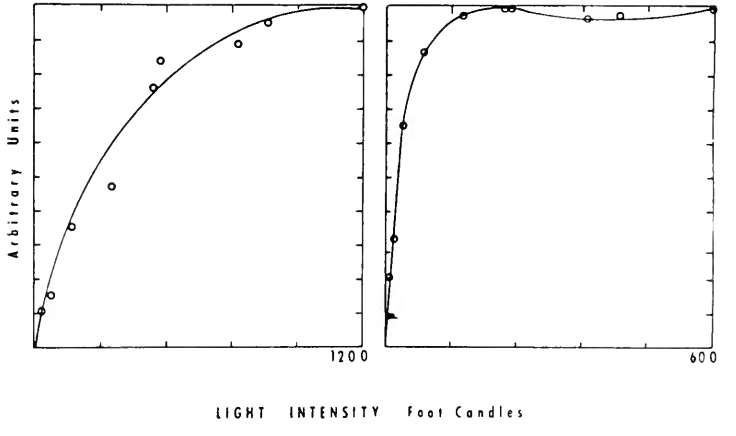


Fig. 2. Light intensity dependence of absorption spectrum changes at 525 mμ during the induction peak (right figure) and at steady state (left figure). Note difference in light intensity scales. Transmission change in arbitrary units. Red light, direct cross illumination.

tensity during the induction maximum (*a*) and at steady state (*b*) The process saturates at different incident intensities during the successive phases of the induction period. In this respect it is strikingly similar to the luminescence.

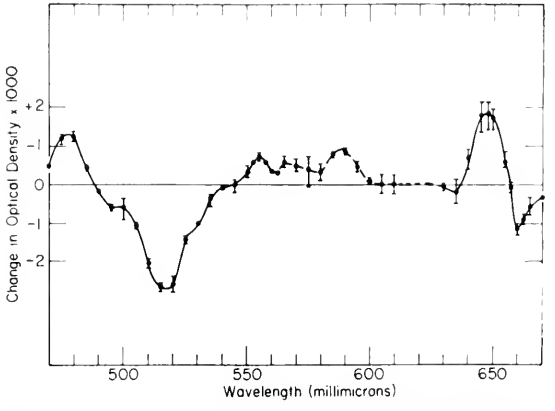


Fig. 3. Difference spectrum of *Chlorella* obtained with a flow system. Illumination with white light of 600 foot-candles intensity. Calibrated at each wavelength against a known transmission change of *ca.* 0.5%.

3. In order to extend the observations into the red region of the spectrum we found it necessary to utilize the flowing systems. Figure 3 illustrates the effect of illumination on the difference spectrum from about 470 mμ to 670 mμ. Note that the bands at 480 and 520 mμ are inverted with respect to the results obtained during illumination. It therefore appears (as is also evident from the time course illustrated in Fig. 1) that immediately following illumination there is a decrease in absorption at 520 mμ and an increase at 480 mμ.

4. Of particular note in Fig. 3 are the pair of strong bands appearing at 648 mμ and disappearing at 660 mμ.

5. Our measurements extended as far as 720 mμ and no changes were observed in the region 670 to 720 mμ greater than 10% of the change at 520 mμ, if there were indeed changes at all in this far red region.

6. When the cells were illuminated for a fraction of a second and then assayed simultaneously for luminescence and absorption, changes in absorption were found to be similar to the changes occurring under direct illumination. Apparently the inversion effect requires at least

several seconds of illumination and a short period in the dark following the illumination in order to appear.

7. In order to determine whether the 520-mμ and 648-mμ changes

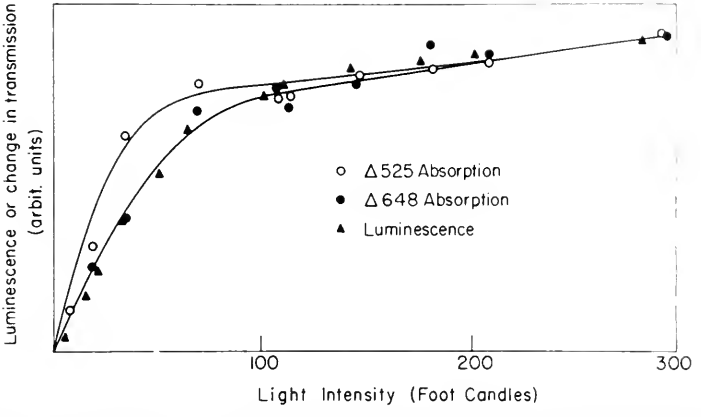


Fig. 4. Light saturation curves for 525 mμ, 648 mμ, and luminescence using a flow system and illuminating the algae for about 0.1 second prior to measurement.

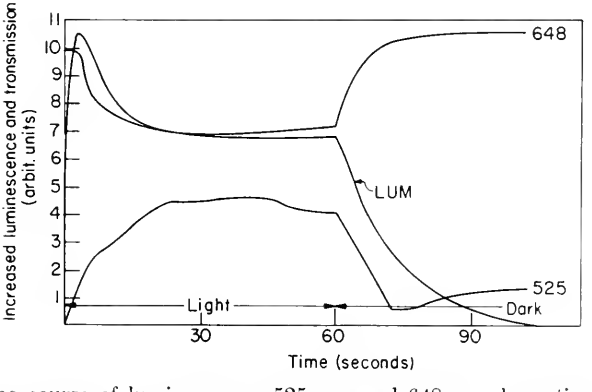


Fig. 5. Time course of luminescence, 525-mμ, and 648-mμ absorption in a flow system.

are due to the same compound, we have measured the dependence of the two processes on illuminating intensity. The intensity dependence appears to be different for these two wavelengths. These results and the time course of the luminescence compared to the absorption spectrum changes are illustrated in Figs. 4 and 5. The luminescence paral-

lels both the 520- and 648-mμ bands in its time course, but appears closer to the 648-mμ band in its response to light intensity variations. It is also clear from these measurements that these two peaks are due to different chemical compounds, since the changes do not parallel each other as a function of the external variables.

8. It was established that the 520-mμ absorption band could not be due to a modulated red fluorescence artifact because there was no

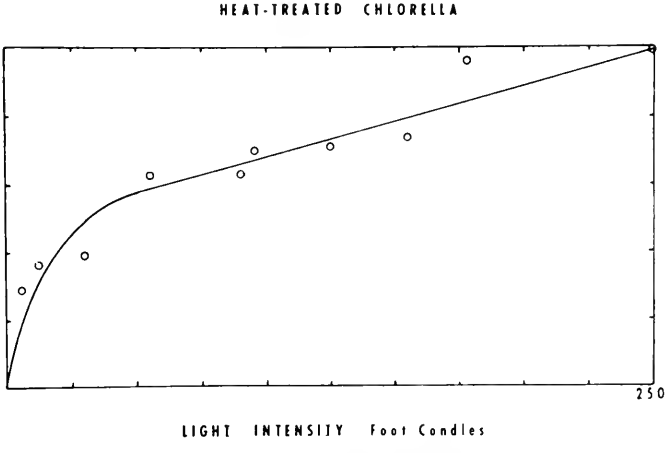


Fig. 6. Light intensity dependence of *Chlorella* transmission changes at 525 mμ following partial inactivation by heat. The deflection equals about 3 to 4 O.D.U. $\times 10^{-3}$ at saturation.

difference signal when a red filter was inserted in front of the photomultiplier and the cells were illuminated in a flow system. The changes could not be due to a modulating green fluorescence because there is little if any green fluorescence in *Chlorella*.

9. When the cells were subjected to heat treatment and then illuminated an interesting effect was noted. After 4 minutes at 51°C. only an increase in absorption was noted; and, surprisingly, this increase encompassed the entire spectral region measured. Figure 6 shows the intensity dependence of the process, and Fig. 7 illustrates the wavelength dependence of this phenomenon. Treatment at 60°C. for 4 minutes completely destroyed any reversible changes in absorption. The luminescence was reduced to about 5% of the normal value by the former treatment and completely obliterated by the latter.

10. We also noted a series of smaller absorption bands in the region 550 to 660 mμ (see Fig. 3), perhaps due to cytochromes as earlier suggested by Lundegårdh.

These results permit the following tentative conclusions:

First, there is formed during illumination a compound with an absorption band around 520 mμ. In succeeding dark periods, its concentration falls to a level considerably below that of the dark value prior to illumination. This compound can be formed in the dark by thermal chemical reactions as well as by photochemistry, since its

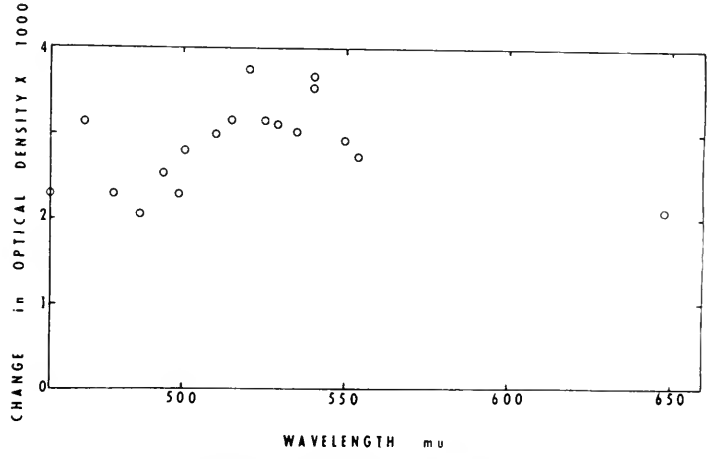


Fig. 7. Change in optical density at different wavelengths during illumination following heat treatment of *Chlorella* for 4 minutes at 51°C. Incident intensity, 250 foot-candles in a flow system. Temperature, 25°C.

concentration rises again to a higher level in the dark after the initial depression.

Second, there is also formed another compound with an absorption maximum around 648 mμ different from the one having the 520-mμ band chemically. Both of these substances appear to be related to the luminescence process since their concentration and the luminescence intensity exhibit similar kinetic behavior and time courses.

Third, it appears unlikely that the 520-mμ band is due to a chlorophyll *a* derivative, since there is no comparable change in the chlorophyll absorption maximum in the red or blue. Rather, it is suggested that this compound may be a flavin-type free radical which is pro-

TABLE I

Worker	Measuring wavelength	Method of illumination	Time of measurement	Half-life of effect	Sign of effect
Duysens	520	Direct (cross illumination)	During illumination	?	+
Witt	520	Direct (cross illumination) flash	During illumination	*Ca.* $^1/_{1000}$ sec.[a]	+
Present	520	Direct	During illumination	Several sec.[a]	+
Present	520	Circulating system —illuminated for 30 sec.	After illumination	Several sec.	—
Present	520	Circulating system —illuminated for *ca.* 0.2 sec.	After illumination	?	+
Duysens	480	Direct (cross) illumination	During illumination	?	—
Witt	480	Direct (flash)	During and after illumination	*Ca.* $^1/_{100}$ sec.	—
Present	480	Direct (cross) illumination	During illumination	Several sec.	—
Present	480	Circulating system —illuminated for 30 sec.	After illumination	Several sec.	+
Present	480	Circulating system —illuminated for 0.2 sec.	After illumination	?	—
Lundegårdh	555	Circulating system	After illumination	?	—
Present	555	Circulating system —30 sec. illumination	After illumination	Several sec.	+
Present	648	Circulating system —30 sec. illumination	After illumination	Several sec.	—
Present	648	Circulating system —0.2 sec. illumination	After illumination	?	—
Present	660	Circulating system —30 sec. illumination	After illumination	Several sec.	—

[a] Note: The *ca.* $^1/_{100}$ sec. half-life is reminiscent of both the Emerson-Arnold time and the short decay luminescence described elsewhere in this volume, whereas the long-lived change in absorption is analogous to the long-lived luminescence.

duced photochemically and is also in chemical equilibrium, in the dark and through enzymatic reactions, with both the photoproduced oxidant and other redox systems in the algae. The 648-mμ band likewise does not appear to be due to chlorophyll *a;* it may be a chlorophyll *b* derivative but it is also possible that it represents some other type of compound, for example, a cytochrome. Possibly it is the photoproduced oxidant, a precursor of molecular oxygen.

Fourth, a partial thermal inactivation produces a change in response characterized by increases in absorption over the entire wavelength region observed. We cannot account for this phenomenon at present.

One cannot say, presently, with any degree of certainty what the compounds involved are. The results reported here and in the earlier literature are tabulated in Table I.

To summarize, the simplest interpretation of the present findings consistent with earlier work on luminescence and absorption spectroscopy is the following:

1. The absorption bands at 480 and 520 mμ are due, respectively, to the oxidized (X) and reduced (XH) states of the same compound. The assignment of the 520 band to the reduced state follows (*a*) from Witt's observations on the effect of added hydrogen acceptors which depress the 520 changes and (*b*) from the fact that the absorption falls below the steady dark level immediately following illumination and then, in the succeeding dark period, rises to the normal dark level. Since it is unlikely that the increase is due to the accumulation in the dark of precursors of molecular oxygen similar to those produced photochemically and since one would *a priori* expect the primary reducing agent to be in dynamic chemical equilibrium with other metabolic pools of hydrogen in the light as well as the dark, it is probable that the 520-mμ band is due to a reduced compound.

2. The 648-mμ band may represent the oxidized form of the precursor of O_2, that is, the photochemically produced oxidant (YOH).

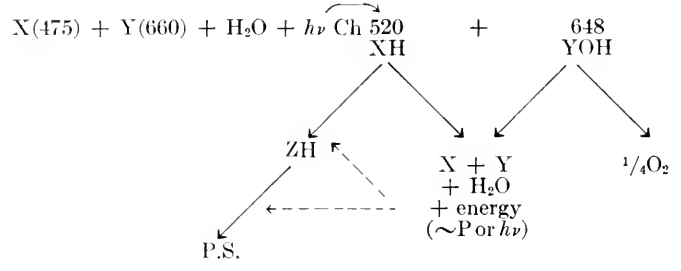
3. During illumination there is always an increase in the concentration of XH over its steady dark value, but in the succeeding dark period it may fall below the dark level at steady state, because XH can be consumed by a concurrently produced oxidant. Such a picture is also adequate to explain the qualitative aspects of the induction effects in absorption spectrum changes and in luminescence.

Such an inequality between reductant and oxidant could arise be-

cause the reductant is presumably being consumed by other hydrogen acceptors in the photosynthetic chemical chain while the oxidant disappears mainly in a reaction producing molecular oxygen. If the rates for disappearance of YOH are such that the steady-state concentration of oxidant in prolonged light is higher than that of the reductant the changes observed would occur.

4. There should always be an increase in reductant during the initial phase of illumination because the reductant and oxidant are approximately equal in concentration at the onset of photochemistry.

These assumptions can explain all the observed results with normal algae. Schematically the relationships postulated are as follows:



Luminescence $\sim$[XH] [YOH]
O_2 Production $\sim$[YOH]^n
Photosynthesis $\sim$[Z] [XH] [A?]

Acknowledgment. This work was performed while the senior author was a visiting investigator at the Carnegie Institution of Washington, Department of Plant Biology, Stanford, California, during the summer of 1955 and was supported in part by a Grant from the U.S. Atomic Energy Commission.

The authors wish gratefully to acknowledge the helpful assistance of Mr. L. R. Kruger in the building and modification of apparatuses and the pertinent and useful guidance and comments of Drs. C. S. French and James H. C. Smith.

Discussion

Brown: Does this hot bath cause any precipitation?

Strehler: Yes, I think so because we got occasional noninstrumental noise pulses, probably due to the interruption of the measuring beam by agglomerates of cells after heating.

Frenkel: I don't quite understand how Dr. Strehler's theory can account for emission. Of course, you assume that the light that is reemitted comes from a substance closely related to chlorophyll.

Strehler: I am quite certain it is emitted from chlorophyll, but that says nothing about whether the energy for exciting it comes from a reaction involving the chlorophyll molecule.

Frenkel: Then you could have a recombination of two molecules other than chlorophyll?

Strehler: I didn't say that both of them were other than chlorophyll, did I?

Duysens: I may perhaps make a suggestion. If reduced riboflavin in the chloroplast tries to emit light, it will be unable to emit it because the chlorophyll traps the excitation energy and you will get chlorophyll *a* luminescence. So it is quite possible there is luminescence of riboflavin.

Strehler: Riboflavin is known to be involved in other luminescences as the emitting molecule. Light emitted by flavin could certainly be trapped by any other absorber present, and what is a more likely absorber in green plants than chlorophyll? I hope you don't get the impression that I have evidence for this hypothesis. I am simply saying that this is another possibility.

Rabinowitch: It sounds to me like why make it simple when it can be made complicated.

Strehler: In support of the hypothesis it has been shown with firefly extracts that added fluorescent dyes in a relatively low concentration change completely the color of the light emitted by firefly extracts. If one chemiluminescent system can do it, certainly another can.

Becker: You can talk about this until you are blue in the face, but I think it is important that you realize you cannot say anything about this energy transfer unless you realize that the molecules that are going to receive it have energy values of the same comparable value.

Duysens: They are there. The fluorescence spectrum of riboflavin overlaps the continuous absorption spectrum of chlorophyll.

Strehler: The riboflavin emission band is quite broad. It extends from about 485 to 620 mμ, and, although this region does not encompass a major band of chlorophyll, there certainly is enough absorption there, particularly if the molecules are in intimate association, to permit energy transfer.

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Selective Scattering of Light by Pigment-Containing Plant Cells*

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We have observed a strong spectral selectivity in the scattering of light by pigmented algal cells. Sharp maxima in the scattering occur on the long-wavelength side of absorption bands (*cf.* Figs. 2, 3, 4).

The experimental apparatus is shown diagrammatically in Fig. 1. The cell suspensions were illuminated with light from a Bausch and Lomb grating monochromator (3.3-mμ band half-width). The intensity of light scattered at approximately 90° (more precisely, 75° to 105°) to the incident beam, I_s, was measured with a photomultiplier tube and was compared with that of the incident light, I_0, by replacing the cell suspension with a white (MgO) surface. The scattered light collected by the measuring device was of the order of 0.1% of the incident light. In the range of cell concentrations used (corresponding to optical densities of ~0.01 to 0.05 per 1-cm. path in the maxima of the absorption bands), the amount of scattering per cell did not vary with cell concentration.

A correction was made for attenuation of the incident and scattered beams in the suspension by cells other than the primary scattering cell itself. This was accomplished by attenuating the incident light in the measurement of I_0 by means of a cell suspension of properly adjusted optical density which was placed immediately before the MgO surface. No correction was made, however, for absorption within the scattering cell itself, since this would require a detailed knowledge of the structure and optical properties of the cell. The asymmetry of the scattering curves in relation to the absorption curves clearly shows, however, that the presence of scattering maxima

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is *not* primarily due to selective absorption of nonselectively scattered light within the scattering cell.

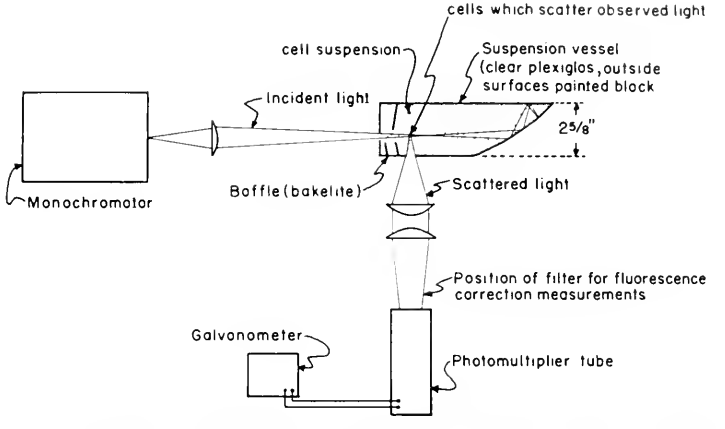

Fig. 1. Arrangement of apparatus for measuring scattered light as a function of wavelength.

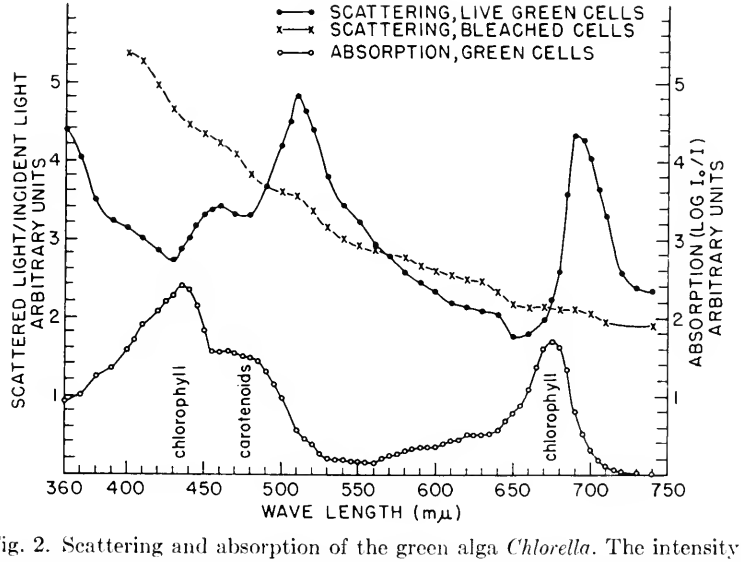

Fig. 2. Scattering and absorption of the green alga *Chlorella*. The intensity of light scattered at 90° to the incident beam is shown as a function of wavelength. Absorption was determined with an integrating sphere. The absorption maxima of the several pigments are indicated on the wavelength scale. The bleached cells were obtained by extraction with hot methanol.

Some pigment fluorescence could be measured together with the scattered light. In spectral regions away from the fluorescence bands, colored glass filters were used to prevent this contamination. Close to and in the fluorescence bands, it was necessary to measure the sum of the scattered light and fluorescence, and to correct for fluorescence. This correction could be based either on the different spectral compositions of the two components or on their different degrees of polari-

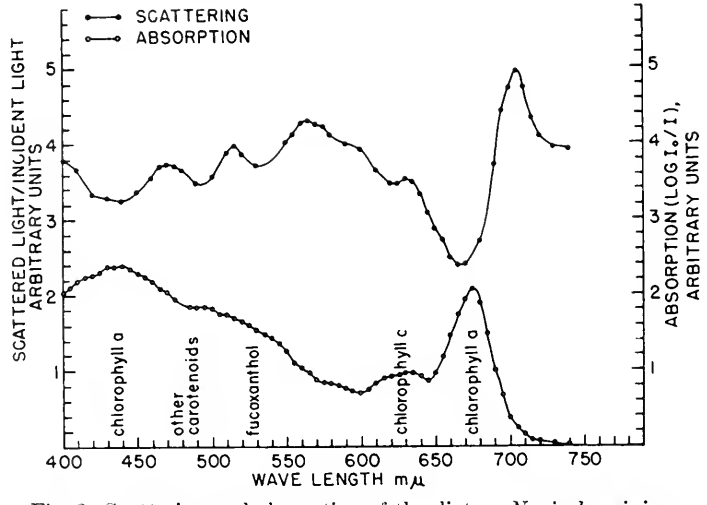

Fig. 3. Scattering and absorption of the diatom *Navicula minima*.

zation. The first method failed near the peak of the fluorescence band; the second had no such limitation.

To determine the fluorescence correction with colored filters, the transmission factor of a filter for scattered light, T_s, was determined at each wavelength with a white surface in the beam, and that for fluorescence, T_f, by exciting the fluorescence with light which appropriate filter combinations could prevent from entering the detector. From the observed average transmission factor for combined scattered light and fluorescence, $T_{s,f}$, the fraction of detector response due to scattering, x, was found by means of the equation:

$$T_s x + T_f(1-x) = T_{s,f}$$

This method of determining the fluorescence correction was used by us with two types of colored filters—one which preferentially trans-

mitted light of *longer* wavelengths (sharp cut-off red filter), and another which transmitted mainly light of *shorter* wavelengths (infrared-absorbing blue filter). Polaroid plates were used in the second method (described by Brice, Nutting, and Hawler (1)).

Corrections found in all three ways were in excellent agreement everywhere except at 680 to 700 mμ, where the different methods led to corrected scattering curves which deviated by up to 10% from the average ones shown in the figures.

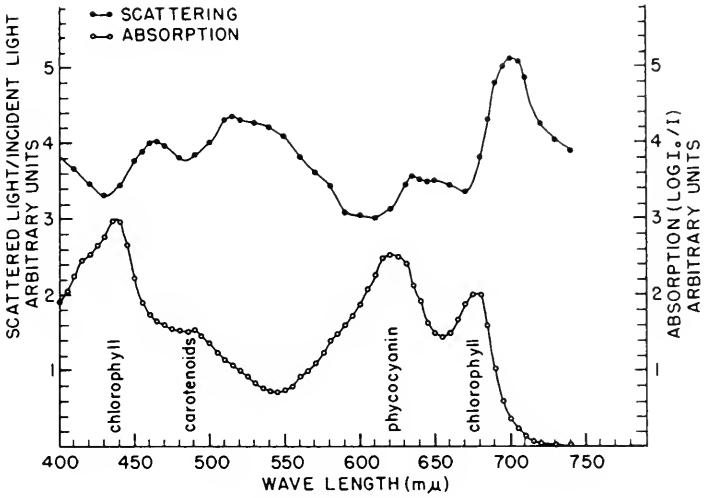

Fig. 4. Scattering and absorption of the blue-green alga *Synechocystis*.

Investigations of crystalline chlorophyll by Jacobs (2) and of suspensions of colloidal chlorophyll by one of the authors had revealed that these systems scatter light with a spectral selectivity similar to that shown in our curves for algal cells.

The light scattered by the cells originates from two sources: (*1*) colorless structures, which scatter with a relatively uniform wavelength dependence (not unlike the scattering by bleached cells, shown in Fig. 2), and (*2*) highly pigmented chloroplasts (or grana), which scatter light with a strong spectral selectivity. The quantitative interpretation of the scattering by pigmented cellular components requires a rather complex theory, such as that of Mie (3). Qualitatively, the capacity to scatter light should increase sharply with the index

of refraction. According to the classical theory of dispersion, the index of refraction of an absorbing medium should reach a maximum on the low-frequency side of an absorption band. This is in fact where scattering maxima have been observed by us.

A similar spectral selectivity has been reported recently by Goedheer (4) and by Menke and Menke (5) in the double refraction of chloroplasts from *Mougeotia*. The quantity which was measured in these cases is the difference between the indices of refraction of the material for light beams polarized in two different planes; scattering depends on the average index of refraction.

The scattering of protons by atomic nuclei shows the same type of selectivity for proton energies in the neighborhood of the resonance levels (6).

Further study of selective scattering may supply information about the packing and arrangement of pigment molecules in biological systems, and may even reveal the presence of pigments which are not easily found by other techniques. As an example, it is seen in Fig. 2 that the selective scattering produced by the carotenoids in *Chlorella* is much more conspicuous than their contribution to the absorption spectra. The latter is so effectively obscured by chlorophyll absorption that the very existence of carotenoids as such in green cells has been doubted (7).

We wish to thank Professor James Franck and Professor Robert Emerson for helpful suggestions and Ruth V. Chalmers for growing the algal cells.

TRANSMISSION CHANGES IN ILLUMINATED CELL SUSPENSIONS

We have heard several reports describing small changes in the transmission of collimated beams by cell suspensions. It may be worth while to draw attention to the fact that changes in scattering, as well as changes in true absorption, could lead to observations of this type.

The figures in the present paper show that, in some regions, light scattered by cells varies sharply with wavelength. The physical structure of a cell, in which the selective scattering originates, may be altered by exposure to light, or by chemical agents. In either case, changes in selective scattering could result and lead to selective changes in transmission. Some investigators have found variations in transmission after changes in temperature (Witt), or after the addi-

tion of HCN (Bishop), which they attribute to changes in scattering. We can also refer to the report of Shibata (see page 171).

The portion of the unabsorbed light which does not reach the detector because of scattering, depends on the geometry of the apparatus and on the absorption of light by the cells. Actually, the contribution of scattering to the total attenuation of the incident beam often is of the same order of magnitude as that of absorption. Therefore, some of the reported variations in transmission, which have been interpreted as changes in absorption, could be the result of equally small relative changes in scattering. Experimental evidence is needed to eliminate this possibility or to evaluate the contributions of absorption and scattering to the observed effect.

Discussion

Weigl: Similar, although somewhat clearer-cut, anomalous dispersion curves have been observed in specular reflection from solid dyes.

Rabinowitch: What particularly interested me in this work is that one gets such strong effects from minor pigments. The example of carotenoids shows that one can detect the presence of some pigments easier by scattering than by absorption. One sees scattering peaks where one doesn't see even a shoulder on the absorption curve. Perhaps, when an adequate theory is worked out, one will be able to make conclusions as to the state of the different pigments—how densely they are packed, etc. I hope, therefore, that others will try to play with this effect, both theoretically and experimentally.

Another remark that I want to make does go back to the first Gatlinburg conference. Three years ago, when Jacobs presented our study of the crystal spectra, Kasha and Commoner attacked his conclusions rather sharply, saying that the band shift we had observed may be due entirely to selective scattering. We were convinced that this was not so, but could not prove it at that time by direct experimental evidence; so the criticism scared us a little and caused us to look more closely into the question of scattering, first in crystal suspensions and subsequently in live cells. We found that our crystal spectra were not significantly affected by scattering, but that selective scattering did in fact occur—both in crystals and in living organisms.

Latimer's study is thus a consequence of the discussions at the preceding Gatlinburg conference.

Chance: Anomalous dispersion effects, of course, are not unique to *Chlorella*. An anomalous band at 370 mμ is well known in erythrocytes, and I think it is largely explained by a rapid change of the refractive index.

With regard to Latimer's remarks concerning the possible role of scattering in measurements of small absorption changes, we have worried about this when measuring intracellular optical density changes. We made light scattering and transmission comparisons, which showed that, in the cytochrome system, the

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effect of scattering is negligible, since the pigment concentration is not sufficient to give steep gradients of the refractive index. We think therefore that our measurements were not influenced by scattering. As a further control, one can add a pigment of known optical properties to the scattering suspension and see if the scattering distorts its spectrum. (We used cytochrome for this purpose.) One can then add a detergent which will clarify the suspension; with whole cells, one may also add lysogen, if they are susceptible to its action. We have done this in every case, and compared the spectra before adding the chelating agent and after, and the spectra before and after adding lysogen. Chemical tests also are of assistance: in *Rhodospirillum*, where phototaxis occurs, we would be worried, if it were not for the fact that we did get similar effects with oxygen as with light, and that the spectral changes observed corresponded to those in isolated cytochromes.

I completely agree with Latimer's remarks when it comes to small transmission changes in highly pigmented particles, e.g., *Chlorella*.

Benson: I think that the technique Dr. Shibata has developed, using opal glass, may be useful in testing for this effect.

Latimer: Instead of using opal glass, which collects *most* of the scattered light, one could also use an integrating sphere, which collects *all* of it.

Frenkel: Dr. Shibata, did your results with opal glass and with the sphere agree?

Shibata: I believe that you can get better results using the opal glass, although the differences are small. The opal glass method requires no special instruments.

James Smith: In very fine suspensions of protochlorophyll in its natural state we got no improvement at all with opal glass. Do you think that this was due to the smallness of the particles?

Shibata: I think so.

Duysens: I should like to remark that Latimer measured light perpendicular to the incident beam. For most dilute suspensions, 95% of scattering is in the direction of the beam.

Latimer: I find the same effect at angles from 45 to 135 degrees.

Duysens: Most of the scattering occurs within 35 degrees (or less) to the incident beam.

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The Absolute Quantum Yields of Fluorescence of Photosynthetically Active Pigments*

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Knowledge of the fluorescence yields of photosynthetically active pigments is important for the understanding of their photochemical activity and the probability of resonance energy transfer between these molecules in the living cell. We have measured the absolute quantum yields of fluorescence of chlorophyll *a*, ethyl chlorophyllide *a*, phycocyanin and phycoerythrin in solution, and of chlorophyll *a* and phycocyanin in the living cell, using the integrating sphere technique (1).

Prins (2) found the quantum yield of fluorescence, φ, of chlorophyll ($a + b$?) in solution to be about 10%, whereas Forster and Livingston (1) found that of chlorophyll *a* to be about 25%. Wassink and coworkers (3,4) reported, for chlorophyll in various organisms, φ values of 0.1% to 0.3%. Arnold and Oppenheimer (5) estimated, for phycocyanin in solution, $\varphi \simeq 20\%$, and in the living cell, about 1.5%. Duysens (6) suggested that the methods used by Wassink *et al.* and by Arnold and Oppenheimer to determine the total fluorescence from the observed sample were unsatisfactory. He estimated that their measurements actually indicate that φ of chlorophyll in green cells is about 1%, and φ of phycocyanin in blue-green cells, about 5%. His arguments suggest that the yield of phycocyanin in solution may be as high as 65%.

Pure chlorophyll *a* and chlorophyllide *a* were prepared by Dr. A. S. Holt. Phycocyanin (from *Synechocystis*) and phycoerythrin (from *Porphyridium*) were purified chromatographically on tricalcium phosphate columns, approximately as described by Haxo, O'hEocha,

* This work was carried out with the assistance of the Office of Naval Research. The paper is based on a dissertation submitted by Paul Latimer in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Graduate College of the University of Illinois, 1956.

and Norris (7). The phycobilin pigments were kept at about 5°C. during the purification and the fluorescence measurements. Fluorescein (Eastman, Reagent Grade) was used without further purification.

The algal cells, upon removal from the culture line, were suspended in carbonate buffer #9 and exposed for $^1/_2$ to 2 hours to visible white light, with an intensity of about 3000 ergs/(cm.^{2} sec.) ($\sim$100 lux). Less than 30 seconds elapsed between the removal of the cells from this light and the measurements—a procedure which eliminated well known transients in the intensity of fluorescence following a dark period. The temperature of the live cells and of pigments other than phycobilins was about 25°C. Prof. Robert Emerson and Mrs. Ruth V.

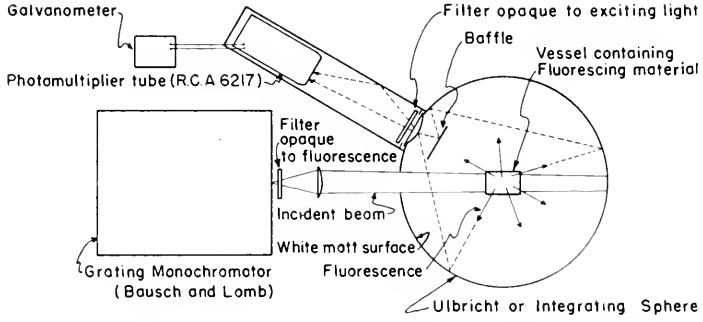

Fig. 1. Arrangement of apparatus for measuring fluorescence.

Chalmers* grew the algal cells and determined the light intensities corresponding to compensation and to one-half saturation of photosynthesis. Different cells were used in the studies of fluorescence and photosynthesis, but they were of the same strain and identically cultured.

The light intensities were measured by means of a photomultiplier tube (RCA 6217): The spectral sensitivity curve of the detector system was determined with a bolometer. By measuring the response of the tube to monochromatic light projected into the sphere, rather than to light falling directly on the tube, we obtained a calibration which accounted not only for the selective sensitivity of the tube but also for changes in the reflectivity of the sphere walls with wavelength. The apparatus is shown in Fig. 1.

As an indirect check of this calibration, we measured the fluores-

* Working on National Science Foundation Contract G-1298.

cence yield of chlorophyll *a* and of phycocyanin in solution as a function of the wavelength of the exciting light. For each of the two pigments, the quantum yields were found to be constant (within ±6%) for excitation between 405 mμ and a wavelength slightly beyond the red absorption maximum. The similarity of the results obtained with the two dissimilar pigments and the agreement of these findings with those obtained by Forster and Livingston in the study of chlorophyll fluorescence may be regarded as indirect evidence that there were no serious errors in the calibration curve.

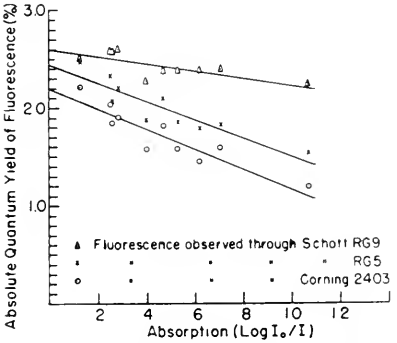

Fig. 2. Fluorescence of *Chlorella* cells. Quantum yield as function of cell concentration. (Log I_0/I is proportional to concentration. Path length of exciting beam = 51 mm.) Yields extrapolated linearly to infinite dilution by method of least squares. Average intensity of exciting light = 50 ergs/(cm.^{2} sec.). $\lambda_{ex.}$ = 436 mμ.

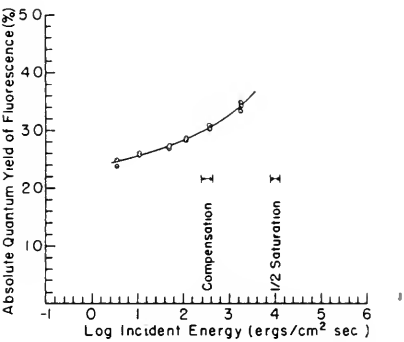

Fig. 3. Fluorescence of *Chlorella* cells. Fluorescence as function of intensity of exciting beam (averaged over its path in vessel). $\lambda_{ex.}$ = 436 mμ.

The average sensitivity of the detector to the fluorescence emitted by the different pigments and cells was determined from the calibration curve of the detector system and the known fluorescence spectra.

In order to correct for the reabsorption of fluorescence, the yields of fluorescence of several suspensions (or solutions) of different concentration were measured, and the results extrapolated to infinite dilution. This method permits an accurate correction for the reabsorption of the fluorescence in *solutions*, but it does not account for self-absorption within the emitting cells in cell suspensions. The latter

TABLE I. Fluorescence Yields of Pigments

Pigment	Solvent or organism	Exciting wave-length, λ (mμ)	Int. of exciting beam, ergs/ (cm.² sec.)	Quantum yield, φ (%)	Investigator
Fluorescein (in solution)	Aq. NaOH	436	~10	91	Authors
"	"	"		79	Forster and Livingston (1)
"	"			80	Vavilov (8)
"	"			>81	Hellström (9)
"	"			85	Umberger and LaMer (10)
"	"			>100	Ghosh and Sen-Gupta (11)
Ethyl chlorophyllide *a* (in solution)	Ethyl ether	436	~10	33	Authors
Chlorophyll *a* (in solution)	Ethyl ether	436	~10	33	Authors
"	"	436	~10[6c]	24	Forster and Livingston
"	"			10	Prins (2)
Chlorophyll *a* (in live cells)	*Chlorella pyrenoidosa* (green alga)	436	50	2.7	Authors
"	"	"	0	1.7 -2.0*s*	Authors
"	"	"	~10⁴	0.15-0.30	Vermeulen, Wassink and Reman (3)
"	*Navicula minima* (diatom)	"	100	2.8	Authors
"	*Nitzschia* sp. (diatom)	"	4 × 10⁴	0.25	Wassink and Kersten (4)
"	*Synechocystis* sp. (blue-green)	"	~50	~1.5	Authors
Phycocyanin—from *Synechocystis* (in solution)	0.1 *M* phosphate buffer pH 6.2	546	~10	53	Authors
Phycocyanin—from *Oscillatoria* (in solution)				~20	Arnold and Oppenheimer (5)
Phycoerythrin—from *Porphyridium cruentum* (in solution)	0.1 *M* phosphate buffer pH 6.2	480	~10	85	Authors
Phycocyanin (in live cells)	*Synechocystis*	546	~50	~3.0-3.5	Authors
"	*Oscillatoria* sp.			~1.5	Arnold and Oppenheimer

s Extrapolated to zero intensity. [c] Estimated by authors.

correction was evaluated by using various sharp cut-off red filters to separate the fluorescence from the incident light, thus obtaining values for fluorescence differently influenced by self-absorption. Three filters of various shades of red were used. Total fluorescence yields were computed by dividing the value obtained with each filter, by the fraction of the total fluorescence transmitted by it (determined from the fluorescence spectrum and the transmission curve of the filter). The three values of the total yield at zero concentration obtained in this way can be in turn extrapolated to zero reabsorption (by plotting them as a linear function of the slopes of the straight lines fitting the three sets of experimental points). We arrive in this way at a value of φ unaffected by all forms of self-absorption, "internal" as well as "external."

The quantum yields of fluorescence of chlorophyll in *Chlorella*, excited with an intensity of 50 ergs/(cm.^{2} sec.) at $\lambda = 436$ mμ are plotted in Fig. 2 as a function of the concentration of the algal suspension. Using the above-described procedure to correct for self-absorption, we obtained for the limiting quantum yield of fluorescence, at this intensity of excitation, a value of 2.7%—about ten times that reported by Wassink and co-workers—confirming the prediction of Duysens. The time available for the migration of excitation energy between chlorophyll molecules is, therefore, ten times as long as it had been assumed to be on the basis of earlier data.

The quantum yield of fluorescence of chlorophyll in plant cells has been generally assumed to be independent of the intensity of the exciting light in weak light, but to increase at high, photosynthesis-saturating intensities (12). We have found, however, that the yield is also a function of the intensity of the exciting light at intensities as low as 0.01 of that required for compensation of respiration by photosynthesis. Figure 3 indicates that fluorescence yield, and thus also the average lifetime of excited chlorophyll in cells, is about 50% longer at compensation than it is at very low intensities. (Similar observations were reported by Brugger at this Symposium.)

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Fluorescence Yield of Chlorophyll in *Chlorella* as a Function of Light Intensity*

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It is known that Franck's explanation of the so-called Kok effect has been based on the assumption that photochemical reduction of intermediates of respiration is involved. The question arises whether there might be a corresponding effect on the fluorescence. In examining the published curves of McAlister, we found that it is uncertain whether the data for fluorescence intensity versus irradiation extrapolate linearly to the origin. It was decided to investigate this point further. We have found that the fluorescence yield is indeed lower in the intensity range below compensation of respiration than in the region between compensation and saturation, but no attempt has been made to find out whether the transition of the yield occurs exactly at compensation. This effect varies in its strength with external conditions, as does the Kok effect. However, unlike the somewhat erratic appearance of the latter, the break of the fluorescence yield in the neighborhood of compensation was visible in all our curves so long as photosynthetic activity was not suppressed.

An incandescent lamp with blue filters was the source of irradiation. Light intensity was varied by inserting neutral density filters in the beam. Fluorescence was measured with a 1P22 multiplier phototube equipped with an appropriate red filter. The photosignal was amplified and recorded. The light-detecting system responded linearly with signal input. To locate compensation and saturation intensities on the illumination scale, a modified Warburg manometer was used with which chlorophyll fluorescence as well as oxygen evolution could be followed simultaneously. In most measurements, the manometer was replaced by a sample holder having the same geometry but fitted with a sintered glass bottom so that various gas mixtures could be passed through the suspension. One-milliliter samples of ~0.02% *Chlorella* were used. These had a transmission (1-cm. cuvette) of 55% at 4400 A and 85% at 7800 A. The algae

* This work was partially sponsored by the Office of Naval Research.

were usually suspended in water, though buffers and other solutions were also used. Water was preferred because it simplified the problem of attaining an equilibrium with the flushing gas and thus facilitated the changing from one gas mixture to another.

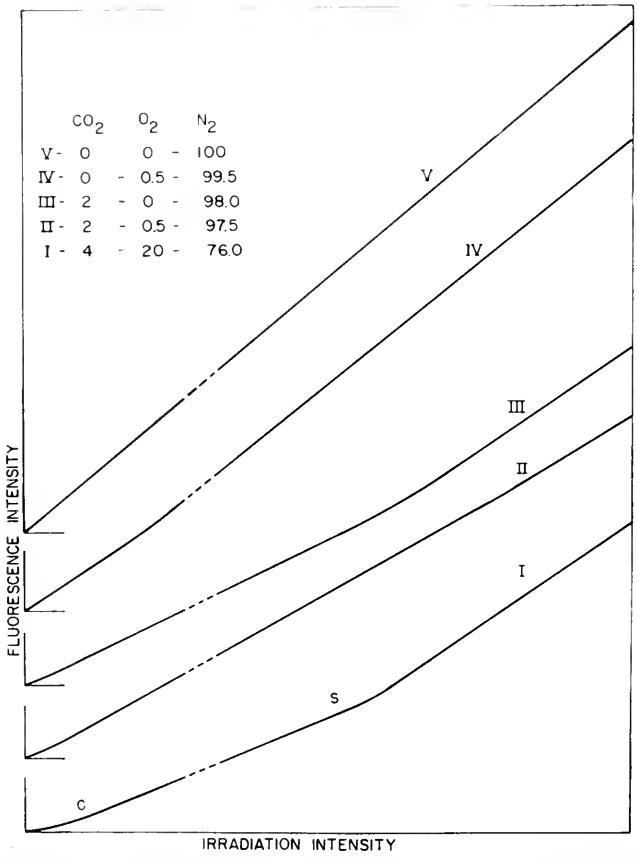

Fig. 1. Fluorescence intensity versus irradiation intensity for *Chlorella* in water swept with various gas mixtures.

The anomalies of fluorescence yield during induction periods, previously reported by others, were observed. However, all measurements reported here are steady-state values. Generally the fluorescence was measured by starting at low intensities of irradiation and

proceeding stepwise to higher ones. When the intensities used were not too great, it was possible to retrace the curve by going from high to low intensities and also to check random points.

Figure 1 shows a fluorescence intensity versus irradiation trace for *Chlorella* in water swept continuously with 4% carbon dioxide in air. There is a break in the curve at low intensities. One observes that

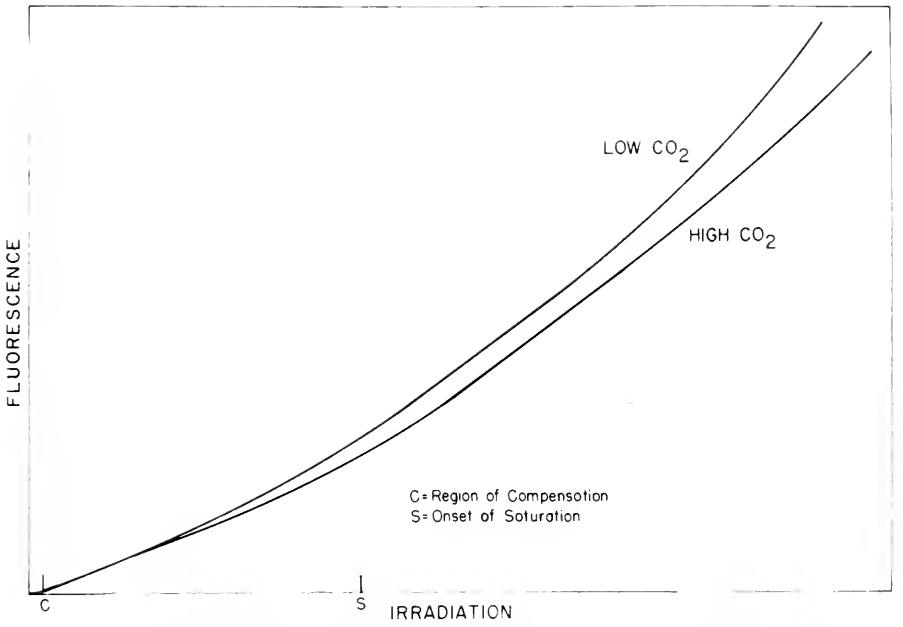

Fig. 2. Fluorescence intensity versus irradiation intensity for *Chlorella* in water swept with ordinary and CO_2-enriched air.

the curve between compensation and saturation, if extrapolated, would pass below zero on the ordinate scale. There is some uncertainty about the exact shape of the curve at low intensities—most probably there is a gently decreasing slope with diminishing irradiation, but a relatively sharp bend in the region of compensation. The slope at approximately half compensation can be seen to be roughly half that of the value between compensation and saturation. Above saturation, the well-known rise of the fluorescence was observed. The slope below compensation was somewhat influenced by the

culturing conditions, previous history of the sample, etc. Algae cultured in a glucose-rich medium showed a lowered initial section of the curve. Algae grown in Knop's solution with ordinary air, as well as those cultured in an NH_4^+-rich medium, showed steeper slopes than the algae grown with CO_2-enriched air. No extended study was made of the effect of the medium in which the algae were suspended during the measurement. Work is being continued along these lines, however. It was found most convenient to store the algae in the dark in carbon dioxide-free air—in this way several experiments could be done on the same batch of algae. For control, the first set of measurements was repeated at the end.

When one compares the curves obtained for 4% carbon dioxide in air with those in unenriched air, he observes that (Fig. 2) the principal difference is the earlier onset of saturation. When the carbon dioxide level is maintained at 4% but the oxygen concentration is reduced (Fig. 1), one observes that the slope at high intensities becomes steeper and the break in the curve occurs progressively earlier. At the same time the initial slope (below compensation) becomes steeper. In the curve (Fig. 1) for 2% carbon dioxide in nitrogen (oxygen less than 10^{-6} mm. Hg), there is essentially a merging of the two breaks observed in the curve measured in 4% carbon dioxide in air. The shape of these curves depends on the degree of anaerobiosis one maintains. It was necessary to use very high flow rates for the CO_2-N_2 sweeping gas. When carbon dioxide is entirely absent but the nitrogen contains 0.5% oxygen, the initial slope is only slightly different from the slope at intermediate and higher intensities. The position of the bend varied with the time the algae were exposed to the gas mixture. In nitrogen containing no carbon dioxide or oxygen (less than 10^{-6} mm. Hg), a linear dependence of fluorescence intensity on irradiation is obtained.

Low temperature increased the fluorescence and straightened the curves. The results obtained under irradiation with green and yellow light did not appear to differ significantly from those obtained with blue light. In addition qualitatively similar results were obtained with *Scenedesmus obliquus* D_3 and *Ankistrodesmus braunii*. Studies of the effects of poisons have not been completed and will not be discussed in this paper. Among those tried—phenylurethane, iodoacetate, cyanide, carbon monoxide—the effects produced were understandable within the framework of the interpretation which follows. In general,

in the presence of poisons, the fluorescence intensity was increased and the bends tended to disappear. A more complete presentation of these experiments will be made at a later time. The theoretical conclusions are incorporated in Dr. Franck's paper on the "Photochemical Part of Photosynthesis" (see page 142).

Discussion

Lumry: I should like to mention some of our preliminary results. We have been trying to find out if the fluorescence yield approaches zero as the light intensity approaches zero. Our measurements suggest that the yield changes with light intensity at much lower intensities than has been previously thought.

Introductory Remarks on the Luminescence of Photosynthetic Organisms*

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In 1951 it was discovered (1) that green plants emit a low intensity chemiluminescence when they are illuminated. This luminescence persists for some time (up to 200 minutes) after the plants are removed from the light (2). Inasmuch as this process undoubtedly represents a minute reversal of early steps in the conversion of light energy to chemical energy and since it is easily measurable, the phenomenon furnishes a useful tool for the study of the photochemistry of photosynthesis. Moreover, a solution of the intermediary chemistry leading to this bioluminescence in all likelihood will also have a direct bearing on the understanding of its converse process, photosynthesis.

The salient features of this phenomenon are as follows.

PHYSICAL PARAMETERS OF PROCESS

1. The emitting molecule is chlorophyll *a*, as is shown by the color of the emitted light, which is identical within experimental error to the fluorescent light emitted (owing to chlorophyll *a*) by green plants (3).

2. The efficiency of different wavelengths of exciting light in producing luminescence parallels their efficiency in promoting photosynthesis (1). The action spectra of green plants (*Chlorella*) parallels chlorophyll absorption, whereas in certain blue-green algae the action spectrum follows that of phycocyanin absorption (4). In this case light absorbed by chlorophyll is inactive (or highly inefficient) in promoting either photosynthesis (5) or chemilumescence, although the emitting molecule is chlorophyll (4).

3. Brown, red, green and blue-green algae and all green plants tested (about 50 different species, including conifers), exhibit the phe-

* This work was supported in part by a grant from the United States Atomic Energy Commission.

nomenon (4,6). It has also been shown that photosynthetic bacteria emit a luminescence of a similar nature at longer wavelengths, although this process has not been intensively investigated (4,7).

4. The intensity of the luminescence is quite low—of the order 10^{-5} to 10^{-7} of the illuminating intensity below saturation, i.e., *ca.* 10^{-3} to 10^{-5} of the fluorescence intensity.

EVIDENCE ON THE ENZYMATIC NATURE OF THE PROCESS

1. The long-lived luminescence increases with increasing illuminating intensity up to a certain value and thereafter remains essentially constant; i.e., it displays a typical saturation as does an enzymatic reaction (1).

2. At unphysiological temperatures the luminescence is reversibly destroyed if the heat treatment is brief. Exposure to 45° to 50°C. for a few minutes irreversibly destroys the luminescence (1).

3. Rate of luminescence as a function of temperature shows a typical enzymatic temperature optimum at *ca.* 37°C. (the temperature optimum for photosynthesis) with a heat of activation for the process of *ca.* 10 to 15 kcal. (1). That this activation energy is characteristic of the luminescent substrate was established by measuring in a flow system the temperature dependence of the emission process after illumination at different temperatures (1). The intensity of light emission was strongly dependent on the measuring temperature and essentially independent of the temperature at which the plants were illuminated (between 0° and 40°C.). That the formation of luminescent substrate does involve some activation energy was shown by illumination at liquid nitrogen temperature, after which no luminescence is observable upon thawing.

4. Various metabolic inhibitors, including U.V. light, produce strong changes in luminescence (1,8,9).

DECAY CHARACTERISTICS

1. The luminescence does not decay according to any simple kinetic formulation (1,2,6). About one-half of the luminescence disappears in 0.1 second, after which it decays more slowly.

2. At least two definite components are distinguishable in the decay curves (see Fig. 1):

a. A fast decaying component of *ca.* 0.01 second duration that

does not appear to saturate at high light intensity (note resemblance to Emerson-Arnold time).

b. A slowly decaying luminescence that reaches saturation at moderate incident light intensity.

3. The time course of the luminescence induction period is different for different portions of the decay curve (6). The long-term

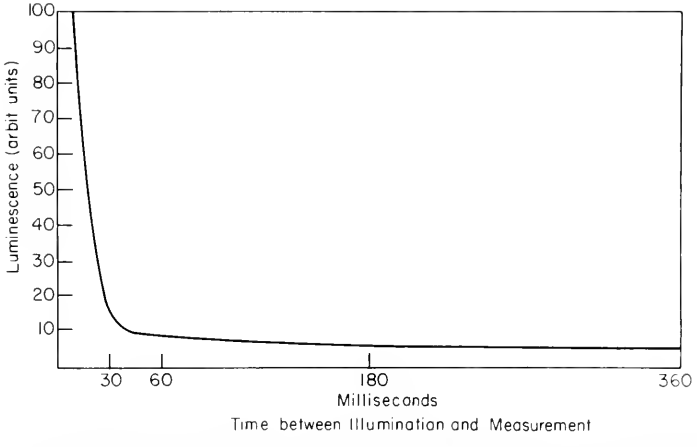

Fig. 1. The decay of *Chlorella* luminescence measured in a phosphoroscope Integrated intensity constant at *ca.* 300 foot-candles. Temperature, 25°C.

decay possesses a marked induction maximum whereas the fast decay component shows a much less marked maximum and actually increases with time of illumination.

RELATION TO PHOTOSYNTHESIS

1. In intact plants the intensity of luminescence is influenced by the presence or absence of CO_2 and by anaerobic conditions (1,9). CO_2 depresses luminescence, as might be expected if it removes photochemically produced reductant. Prolonged exposure to anaerobic conditions inhibits the luminescence (9).

In a parallel manner, the luminescence of chloroplasts is inhibited by the addition of hydrogen acceptors such as the Hill oxidants, ferricyanide, or quinone (6,9).

2. The luminescence is also exhibited by chloroplasts and saturates parallel to the Hill reaction rate as a function of incident light inten-

sity. The ability of chloroplasts to luminesce disappears during prolonged storage of chloroplasts at −20°C., as does the Hill reaction. However, lyophilized powders retain activity apparently indefinitely.

3. With the exception of a gradual increase in the level of luminescence under certain conditions during the first few minutes of illumination chloroplasts do not exhibit any induction effects (6). By contrast, the luminescence of intact plants evidences striking fluctuations in intensity during the first few minutes of illumination (1,9). These induction effects are very similar in their time course to a number of other transients in photosynthesis, e.g., fluorescence (10,11), ATP concentration (12,13), O_2 liberation (14), and CO_2 fixation (see Fig. 2).

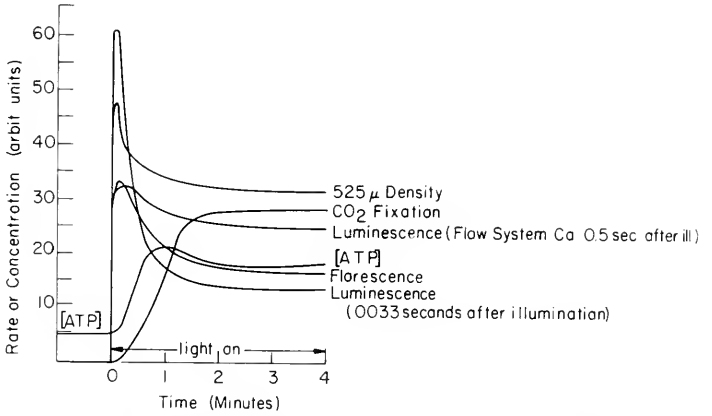

Fig. 2. A comparison of induction curves for various processes connected with photosynthesis. Note that the time required for steady state to be reached is between 90 and 120 seconds for all these processes. The ATP curve represents concentration, not rate of turnover. The CO_2 curve, on the other hand, is calculated from the slope of the total fixed $C^{14}O_2$ as a function of time of illumination (Strehler, unpublished experiments).

These facts strongly suggest the following conclusions:

1. The luminescence of green plants is due to an enzymatically catalyzed recombination of early photoproducts in photosynthesis, probably the primary reducing and oxidizing agent or the very next reactants in the sequence of O_2 liberation and CO_2 reduction.

2. Conditions which would be expected to cause an accumulation of these photoproducts increase the luminescence and, conversely,

conditions which should promote utilization or decrease production of these products decrease the intensity of luminescence.

3. The induction curves are due to changes in concentration of a number of intermediates in the chain of reactions leading from the photochemical events to CO_2 fixation, including the formation and utilization of ATP.

4. The decay curves suggest that two molecular species (probably on the reductant side) are capable of eliciting luminescence. The short-lived component may be regarded as the primary photoproduct and the longer lived component as a substance derived from the first and representing a later hydrogen or OH carrier in the photosynthetic sequence. It is interesting that the short- and long-lived components behave kinetically similarly to the various constants derived from flashing light experiments and thus mixtures of different lifetime intermediates may be responsible for the discrepant results reported by various authors (15–18) under differing conditions of illumination, flash duration, etc.

Acknowledgments. I wish to acknowledge with thanks many stimulating discussions with Drs. William Arnold, James Franck, and Hans Gaffron on the subject material here summarized. It should be emphasized that some of the detailed interpretations here set forth are in conflict with parallel interpretations of Drs. Franck and Brugger of this laboratory, although we are in agreement about the general nature of the process.

Discussion

Rabinowitch: Does this mean that one curve is first order and the other curve is zero order?

Strehler: The best information (Arnold's work) would indicate that, with a very brief flash, one obtains a second-order decay. But, when one uses a longer flash, he obtains something that is neither first nor second order but in between.

Wassink: I would like to ask just one question; namely, how does any consideration of the triplet state of the chlorophyll come into this?

Strehler: I don't think it is necessary, on the basis of the information we have, to postulate anything about triplet states. I think that should await a better understanding of what the natures of the intermediates are.

Wassink: If a triplet state is involved, it must come in between your chemistry and luminescence, just as it must for fluorescence.

Strehler: I believe so—yes.

Wassink: You don't see any objection?

Strehler: I see no objection, but I probably am not qualified to make the judgment.

Bassham: I would like to ask a couple of questions. First, what was the effect of oxygen, if any, on the chemiluminescence? Second, does the heat of activation refer to the long component or to the short component or to both? Third, can the short component possibly be, rather than chemiluminescence, a long-lived metastable state of some kind in the pigment?

Strehler: I am not sure it is possible to give a definite answer to any one of these questions. Arnold and I did not find an appreciable effect of anaerobiosis on luminescence. After incubation of the algae for some time under extreme anaerobic conditions, however, interesting effects have been observed, as shown by Brugger and Franck. We measured the heat of activation for the long component. Certainly the short component could be due to a long-lived metastable component.

Linschitz: Two comments. First, if this luminescence is actually due to some metastable product, I claim the product can not be a molecule in the triplet state, since the radiative lifetime of the triplet would not be of the proper order of magnitude. Second, there may also be an activation energy for the formation of a metastable product. This activation energy may possibly be associated with the falling off of the quantum yield on the long-wave side of the absorption.

Strehler: But, as you pointed out at one time, the sharpness indicates that there is not too much activation energy involved.

Linschitz: That is right.

Lumry: I wonder if there is any forward reaction or any other reaction by which you could lose high-energy forms.

Strehler: Inasmuch as the yield of fluorescence is of the order of 1% of the light absorbed, excited singlet states derived by any other mechanism would probably have the same probability therefore of emitting light, namely 1 in 100. The rate of the reaction that we are studying is probably of the order of 100 times greater than what we deduce directly from the chemiluminescence intensity.

Linschitz: In other words, you are saying that if there are any other reactions they are small compared to the reaction—

Strehler: No, I would not say that. I think that all of the reactions involved in photosynthesis which make use of the same intermediates would have an influence on the concentration of luminescent intermediates and therefore on the yield of luminescence. There may also be recombinations of primary oxidant and reductant at other sites that do not lead to luminescence.

Linschitz: I was trying to find out what the activation energy means here. If you have other non-luminescence-producing processes leading to a destruction of your high-energy substances, then the activation energy becomes related to the luminescence in a complicated way.

Strehler: You have emphasized a serious objection to glib interpretations of activation energy calculations.

Rabinowitch: I wonder how long the fluorescence due to return from the triplet to the singlet state survives. If the activation energy—that is, the difference in energy between triplet and excited singlet—is about 10 kcal., then one can calculate how much emission there should be when molecules in the phosphorescent state return to the fluorescent state. Is this "delayed emission" large enough to actually be a component of luminescence? Of course, the luminescence can be reabsorbed by the chlorophyll and thus tend to maintain the concentration of

metastable states—a partly self-perpetuating reaction which involves no chemistry.

Strehler: That is certainly a possibility. The only thing about trying to determine the rate of back reaction is that one has to assume that the fluorescent yield from all chlorophyll molecules is identical. It may be that the very molecules that are involved in the photochemistry have a much different quantum yield for fluorescence than do the average—higher or lower.

Rabinowitch: Still, did you ever look whether red phosphorescence occurs in chlorophyll solutions?

Strehler: We have never been able to detect with the quantum counter a luminescence of chlorophyll in anaerobic solutions after illumination. There is a chemiluminescence of chlorophyll in solution which I will mention, very briefly, later in this session. This luminescence does have a longer lifetime but is probably only of interest from a chemical standpoint.

Weigl: If the reactions of this primary product are enzymatic, as they almost surely must be, it ought to be possible somehow to find a specific inhibitor for one or another of the steps and to increase the luminescence by adding inhibitors. Have you had any luck with that?

Strehler: Cyanide, azide, phenylurethane—a variety of substances increase the luminescence up to almost twofold before they begin to inhibit. We have made the argument earlier that this is consistent with the idea of the pile-up of intermediates. Hydroxylamine has a specificity of action which most of the other inhibitors do not have, in that it seems selectively to depress the long-lived luminescence but to inhibit the short-lived luminescence to a much lesser extent.

Gaffron: I remember that at least in the first experiment with hydroxylamine your concentrations were rather high. Do you have one in the physiological range?

Strehler: Yes, hydroxylamine is effective at around 10^{-5} mole in intact *Chlorella* for inhibiting the long luminescence.

Frank Allen: What is the possibility that this luminescence might be coupled with the decomposition of peroxide?

Strehler: I think that the primary oxidant might be something perhaps analogous to a peroxide. One just can't answer your question until one has the system in extract.

James Smith: If the luminescence spectrum is the same as the fluorescence spectrum of chlorophyll, doesn't this practically eliminate the need to look for a pigment that absorbs at longer wavelengths than chlorophyll in the radiation transfer?

Strehler: Except for antistokes sensitization.

James Smith: It seems to me that, if the reaction products react and re-activate the chlorophyll, it must be the end of the line so far as the radiation transfer is concerned.

Strehler: I think that is a good argument. What do you think, Dr. Arnold?

Arnold: I have thought about it. I am not sure that a triplet state of chlorophyll is not produced by the back reaction, and that the triplet crosses over into an excited singlet which emits the luminescence.

James Smith: If a pigment other than chlorophyll were excited in the back reaction, wouldn't you get a luminescence of this pigment?

Arnold: It is difficult to say that you do not, because there are no photomultipliers sensitive for long wavelengths. Suppose it is considerably far into the red. The curve that Davidson and I published, giving the identity of the spectrum from the delayed light and the fluorescent light, was submitted to our statistical analysis panel in Oak Ridge. If you use one test, the two curves are certainly the same. If you use another test, they are systematically different and there is a little radiation in the red.

James Smith: This seems to me to be a very important thing to determine.

Arnon: I was interested in your remarks about the longevity of this effect in various chloroplasts and in your comment that lyophilized chloroplasts retain this activity but the freshly prepared chloroplasts do not. That seems to fit with the observation that, if lyophilized chloroplasts are kept for a long time, only a small activity in the Hill reaction is observed. In your case, if I followed it correctly, the luminescence effect accounts for about 1% or perhaps less of the radiation energy absorbed.

Strehler: Much less than that.

Arnon: Then, would you not require a large level of photochemical activity retention by the chloroplast preparation in order to observe the effect?

Strehler: As a rough estimate, I would say that the yield in the lyophilized preparations is not less than $^1/_4$ of what reasonably fresh chloroplasts emit.

Arnon: I think the point raised by Dr. Rabinowitch, as to why this effect persists, could be explained by the fact that you need only a portion of the photochemical level of activity, and only a small portion at that, in order to observe the effect.

Now, turning to questions on the physiological side, have you ever tried to increase this light emission? As I understand, you have a certain proportion of the light which is not being absorbed or utilized, but thrown back. You work in the absence of any normal electron acceptor, that is, without any CO_2 fixation reaction and in the absence of Hill reagents.

Strehler: In the chloroplast studies, yes.

Arnon: Then, you are re-emitting a very small portion of the absorbed light energy, while the chloroplast has a much higher capacity.

Rabinowitch: How about oxygen? Isn't oxygen a Hill reagent?

Strehler: The preparation may be reducing and thus exchanging oxygen.

Arnon: Can you increase this percentage of radiant energy that is thrown back by adding electron acceptors? Of course, if you have oxygen you are providing a sink for electrons. Can you increase it in some other way? It seems to me it would be a very interesting effect.

Strehler: If you have any ideas on how it might be done, we will be glad to try them.

Wassink: Just one additional comment on the discussion between Dr. Lumry and Dr. Strehler, which I think is also pertinent to a comment that Dr. Arnon made. Is it true that the properties of this chemiluminescence are very much the same as the fluorescence both in the time course and in their relations to environment?

One would expect that any sort of agent that would increase fluorescence might also be expected to increase chemiluminescence.

Strehler: There are some agents, for instance hydroxylamine, that increase the fluorescence only slightly but almost completely destroy the long-lived luminescence. Luminescence is primarily distinguished from fluorescence kinetically in that it saturates as a function of intensity.

Rosenberg: With respect to the point that Dr. Smith raised, do you think it would be fair to say that the luminescence data are neither more nor less convincing than the fluorescence data themselves for the definition of chlorophyll as being a molecule at the end of a chain? We have known from fluorescence data all along that this is presumably the one, and we would be very embarrassed if we found that the luminescence has a spectrum further to the red. I thought possibly if there were another pigment further at the end of the chain beyond chlorophyll it would perhaps be unprofitable to have a fluorescence spectrum so close to that of chlorophyll that one could not distinguish them.

Arnold: This test for identity is very good. They are very similar. If there is extra light to the red of the chlorophyll fluorescence band, it is quite dim and quite close to it.

Rosenberg: The point I was trying to make was that even if they are identical—and I certainly have no doubt from your data that they are—people who are looking for an extra pigment will say, "Well, we really don't know what chlorophyll fluorescence should look like in the cell and perhaps it is not really chlorophyll fluorescence that everybody has been speaking of."

Arnold: It is certainly unproved from the experiments that have been done.

James Smith: May I make just one comment on this? You could get chlorophyll fluorescence out of the cell and still not have it at the end of the chain because in the red algae, as Dr. French and his collaborators have shown, you have fluorescence of the phycobilins even though you have fluorescence from the chlorophyll. So not all of the energy is transferred. Is this not right, Stacy?

French: Yes.

James Smith: I should think you might run into the same situation with chlorophyll itself and another pigment that could receive a fair share of the energy.

Kamen: Dr. Duysens, I remember that in the original work you had a transfer to another pigment near the long wavelength band of chlorophyll. Is that correct?

Duysens: In the fluorescence spectrum of the red alga *Porphyra lacineata*, there is a very strong fluorescence at 730 mμ. If the fluorescing pigment is a concentration of only 0.1% of that of chlorophyll, as we thought, then this fluorescence probably occurs by transfer from chlorophyll *a* to this pigment. There is some, although less clear-cut, evidence for fluorescence at 730 mμ in other red and blue-green algae, but not for green or brown algae.

Kamen: I was wondering whether there is a possibility of a trace amount of another pigment.

Linschitz: 8600 A is the spectral region for the triplet. There may be activation at that level.

Arnold: I have looked very hard for this 8600 A light without finding a trace of it.

Strehler: With respect to what Dr. Kamen had to say, suppose there is as a terminal acceptor an iron porphyrin whose absorption is relatively close to that

of the fluorescence emission of chlorophyll. Since iron porphyrins are relatively nonfluorescent you would not expect to pick up the fluorescent light or any singlet excited state from this compound anyway.

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Decay of the Delayed Light Emission in *Chlorella**

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The process of photosynthesis seems to be partially reversible; green plants emit light for some time after they have been illuminated. Strehler (this volume) has given reasons for believing that this

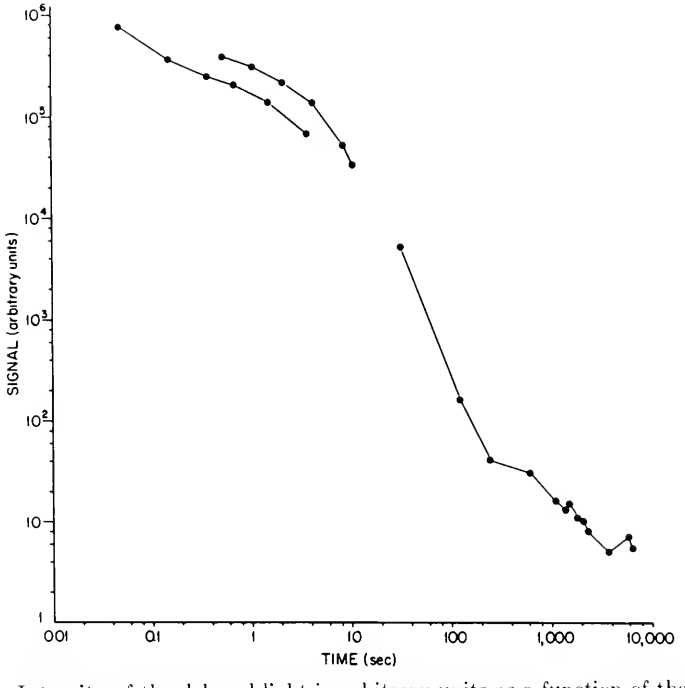

Fig. 1. Intensity of the delayed light in arbitrary units as a function of the time in the dark in seconds.

delayed light production is the reverse of photosynthesis. The present paper is a study of the decay of the delayed light in *Chlorella*. In

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general, no simple relation has been found between the intensity of the delayed light and the time in the dark; for this reason, the results will be presented only in graphical form.

All measurements of the intensity of the delayed light were made with an RCA #6217 Photomultiplier, at the temperature of dry ice,

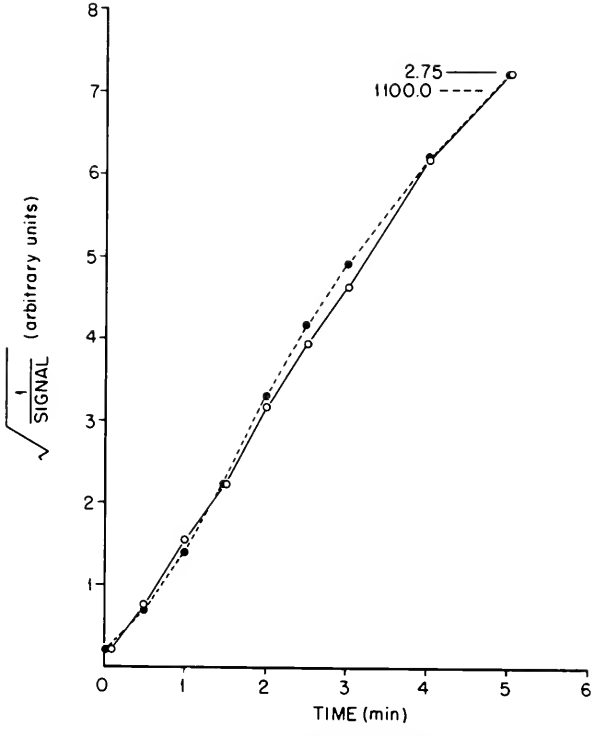

Fig. 2. The reciprocal of the square root of the intensity of the delayed light as a function of the time in the dark. Numbers on the curve indicate relative integrated light energy of the exciting flash.

and with a vibrating reed electrometer. Since the decay covers a wide range of time, three different methods of study were used.

For the middle part of the decay curve the flowing method described by Strehler and Arnold (1) was used, where the time in the dark is the time that the *Chlorella* spends in flowing between the illuminated vessel and the measuring vessel. Each cell spends most of the time in the light, making periodic excursions through the pumping system.

For the last part of the decay curve, the *Chlorella* suspension was transferred after being illuminated into a large glass vessel directly in front of the photomultiplier, and the signal was recorded as a function of the time. The suspension was kept at room temperature by a water jacket. A hand-operated shutter made it possible to check the dark current of the photomultiplier from time to time.

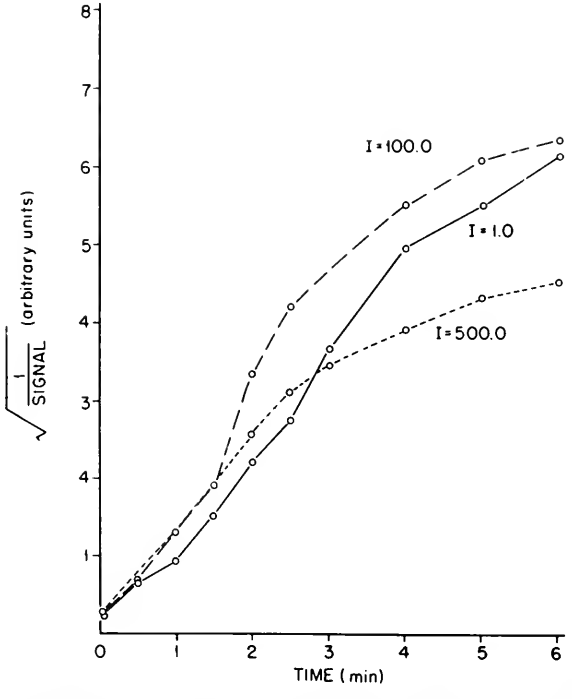

Fig. 3. The reciprocal of the square root of the intensity of the delayed light as a function of the time in the dark. Numbers on the curve give the relative intensity of the continuous exciting light.

For dark times less than a few hundredths of a second, the flowing method is not satisfactory. In order to obtain some information about the beginning of the decay curve a small air-driven high-speed centrifuge was converted into a Becquerel Phosphoroscope. This instrument has two shutters on the same shaft. Each shutter is open a fifth of the time. The two shutters are exactly out of phase. A *Chlorella* suspension placed between the two shutters and illumi-

nated through one of them is excited by flashing light; the number of flashes per second is given directly by an electronic tachometer. Delayed light emitted by the suspension, after passing through the second shutter, falls on the photomultiplier. The signal thus obtained is proportional to the intensity of the delayed light one half-period after the flash. The results are complicated by the fact that the dark time cannot be changed without changing the duration of the flash.

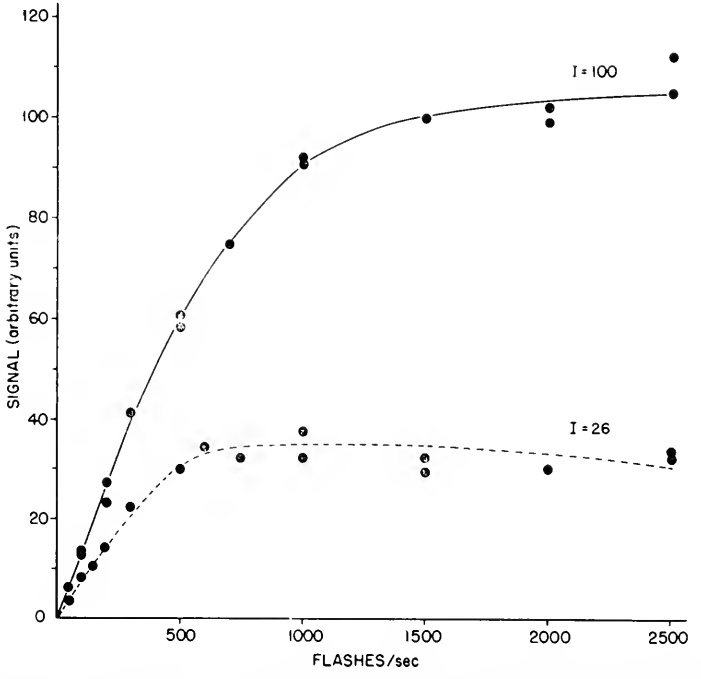

Fig. 4. The intensity of the delayed light as measured with the phosphoroscope as a function of the number of flashes per second. Numbers on the curve are relative exciting light intensities.

Decay curves for the delayed light, at room temperature, between 0.05 and 6000 seconds are given in Fig. 1. The results are plotted on logarithmic scales owing to the wide range covered both as to intensity and time.

There is one exception to the statement that no simple expression has been found for the intensity of the delayed light as a function of

the time in the dark. A *Chlorella* suspension that has been in the dark for 20 to 30 minutes and then exposed to one single flash of light (from a photographic flash bulb) gives over the region of 10 seconds to 10 minutes, a simple second-order decay curve (Fig. 2). However, if the single flash is replaced by continuous light for a few minutes, the results are quite different (Fig. 3).

The intensity of the delayed light, as measured with the phosphoroscope (Fig. 4), increases with increase in the number of flashes per second up to 500 to 1000, and then becomes independent of the frequency of flashing. It may be that this break in the curve can be associated with the new fast reaction in photosynthesis shown by Kok (this volume) in his kinetic studies.

Throughout the entire decay curve for the delayed light, the intensity of the exciting light needed to give maximum signal is increased as the time in the dark is decreased until, as the two curves in Fig. 4 show, at the shortest dark times used ($^1/_{5000}$ second), there is no evidence of saturation.

Discussion

Weigl: The second-order decay after flash illumination suggests the electron-hole recombination kinetics commonly observed in crystal phosphors and photoconductors. Debye and Edwards (*J. Chem. Phys., 20,* 236 (1952); *Science, 116,* 143 (1952)) and Yastrebow (*Doklady Akad. Nauk. SSSR, 90,* 1015 (1953)) have reported similar afterglow in a variety of organic compounds, including proteins. Recombination appears to be limited by the rate at which electrons return to their radicals, and emission takes place by way of the lowest *triplet* state of the parent molecules. In a chlorophyll-protein complex, recombination at the protein could conceivably sensitize luminescence of the dye—even though the observed emission comes from the lowest *singlet* level of the chlorophyll.

Arnold: And an electron takes a very long time finding the radical.

Weigl: Electron *diffusion* can give rise to lifetimes of many minutes or even hours, but only at about −196°C. Deep electron traps or inefficient recombination would have to be demonstrated to make this mechanism seem plausible at room temperature.

Rosenberg: The use of a single flash seems to have simplified the kinetics of recombination.

Arnold: For the longer times.

Rosenberg: In chloroplasts the situation may be easier to investigate where we don't have induction phenomenon and we don't have pools of intermediates that have to be brought up. Is the kinetics simpler for the chloroplast situation?

Arnold: I checked the question of whether or not the saturation depended on the time afterwards that you measured, and it is much the same for chloroplasts.

But I must emphasize what Strehler said about the transients. Whatever these complicated transients are they depend on the whole photosynthetic system much more than the Hill reaction.

Rabinowitch: I was rather confused by the different saturation curves. Can one not suppose that one corresponds to the long component and the short component of different saturation curves and that the others are combinations?

Arnold: Well, I am not convinced that it is justified to break my data into two components.

Rabinowitch: Now, the second question or suggestion. Perhaps some of this complicated law of decay can be associated with the following: You get two products, H and YOH. If both disappear at the same rate, you have a simple second-order reaction. If either one is pulled away faster than the other, you are going to get something much more complicated and you have no reason to assume that the two are pulled out at the same rate, one by the peroxide—

Arnold: Particularly after the plant has been doing photosynthesis in a steady way.

Rabinowitch: Yes, so that seems to be a bimolecular reaction.

References

1. Strehler, B. L., and Arnold, W. A., "Light production by green plants," *J Gen. Physiol.*, *34*, 809–820 (1951).

Some Observations on the Chemiluminescence of Algae*

JOHN E. BRUGGER, *University of Chicago (Fels Fund), Chicago, Illinois*

Our investigations of the chemiluminescence of algae were initiated several years ago. At that time Dr. Rosenberg and I were looking for a phosphorescence of chlorophyll dissolved in rigid glasses or adsorbed on a surface. In the course of this work the chemiluminescence, previously reported by Strehler and Arnold, was observed. It was decided to investigate this phenomenon in a manner somewhat different from that employed by them. Instead of a flow technique, we used a phosphoroscopic method. The results are interestingly different in many respects.

Algae were irradiated with repeated pulses of blue light. Between these flashes, the chemiluminescence was measured. The time sequence was (in milliseconds): irradiate, 1.25; dark, 0.75; measure, 1.25; dark, 0.75. The intensity of the luminescence was determined with a 1P22 multiplier phototube, fitted with a red filter. The photosignal was amplified and recorded (GR DC amplifier and Esterline Angus recorder). At the present time, a quantum counting technique is being used to meter the photosignal. A 3-ml. sample of algae (density: 2 μl. packed wet cells/ml. suspension) was placed in a cell having a sintered glass bottom through which various gases could be passed—another variance from the technique of Strehler and Arnold. *Chlorella pyrenoidosa* were used in the work reported here. However, experiments with *Scenedesmus obliquus* D_3 have given similar results. Irradiation was also undertaken with green, yellow and red light (with and without admixtures of blue light). Within the uncertainties introduced due to light leak and other experimental difficulties, the results are qualitatively the same as those obtained with blue light. As Arnold and

* This work was aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Chicago (Contract ONR 432-(00)).

co-workers have shown, the spectral distribution of chemiluminescence is experimentally indistinguishable from that of the fluorescence of chlorophyll but is several orders of magnitude less intense. The curves presented are not decay curves in the accustomed sense. They are graphs of the intensity of chemiluminescence followed during a period of irradiation—with rapidly pulsed light.

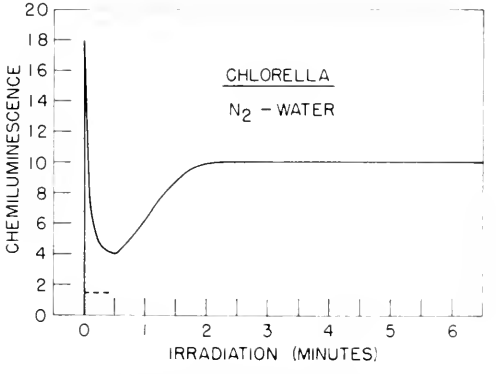

Fig. 1. Time course of delayed luminescence during irradiation of *Chlorella* in water. Nitrogen atmosphere.

The *Chlorella* were frequently suspended in water. This reduced the problem of equilibration with the gas used in sweeping and facilitated rapid changes of sweeping gas composition. Experiments were also conducted using various buffer mixtures to make certain that the aqueous medium introduced no artifacts. Figure 1 shows the variation of the intensity of chemiluminescence during the course of the irradiation. *Chlorella* were suspended in water and swept with oxygen-free (less than 10^{-6} mm. Hg) nitrogen. The rapid initial rise is followed by a deep minimum after which the luminescence rises to a steady-state intensity, which could be maintained for a considerable period of time. The dotted line at about intensity 1.5 indicates background and stray signal. Figure 2 shows the chemiluminescence curve for *Chlorella* suspended in nutrient and swept with 2% carbon dioxide in air. Irregularities such as those observed at 10 to 20 seconds are customarily found. The chemiluminescence then falls to a very low level. The ratio of the initial spike height to the steady-state level is 10 to 15:1.

In the case of *Chlorella* in water swept with pure oxygen, we obtained the curve shown in Fig. 3. Note that the steady state is similar in intensity to that observed in pure nitrogen. Addition of small amounts (short bursts) of carbon dioxide to the flowing gas tem-

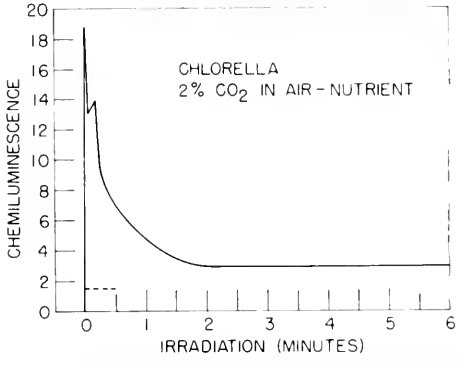

Fig. 2. Time course of delayed luminescence during irradiation of *Chlorella* in nutrient solution. 2% carbon dioxide in air atmosphere.

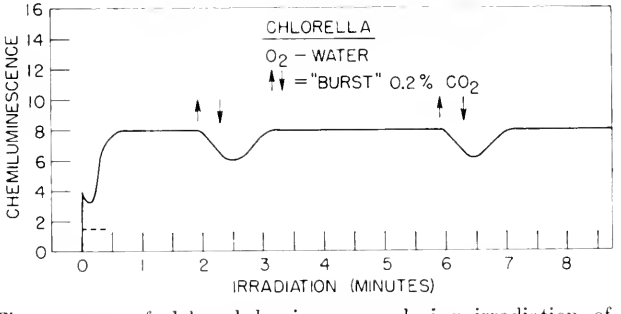

Fig. 3. Time course of delayed luminescence during irradiation of *Chlorella* in water. Oxygen atmosphere. Effect of transient addition of carbon dioxide.

porarily reduced the chemiluminescence intensity, which then quickly returned to its previous value. There was no change in pH of the solution due to the burst of carbon dioxide. The measurement was made by adapting the sensitive pH meter of Professor Gaffron. Something different was observed, as shown in Fig. 4, when a short admixture of carbon dioxide was made when the streaming gas was pure nitrogen. The luminescence was at first depressed but it later rose

to a higher level than it had maintained previously. It was not possible to make the luminescence rise indefinitely with successive bursts. In Fig. 4 it will also be observed that the initial portion of the curve is different from that of Fig. 1. The carbon dioxide effect was demon-

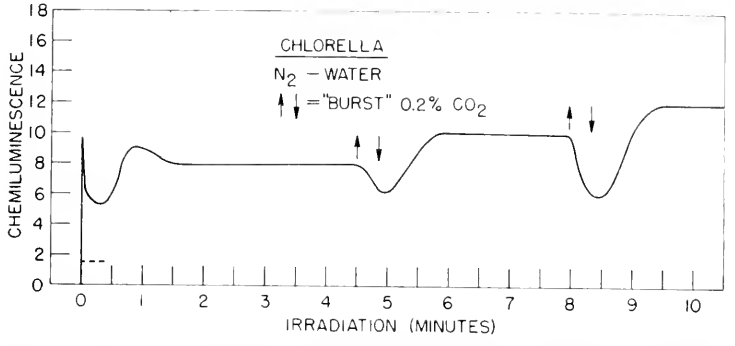

Fig. 4. Time course of delayed luminescence during irradiation of *Chlorella* in water. Nitrogen atmosphere. Effect of transient addition of carbon dioxide.

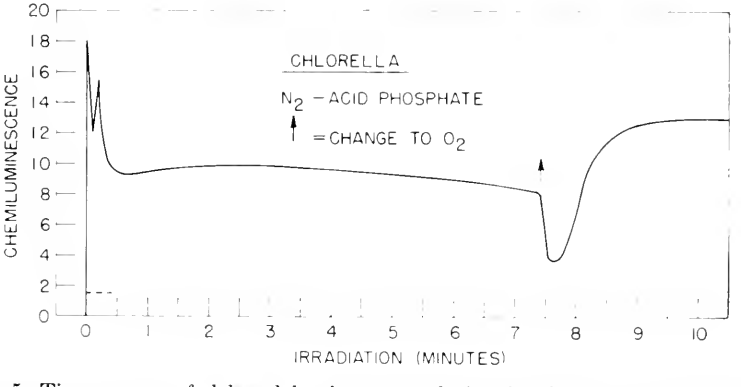

Fig. 5. Time course of delayed luminescence during irradiation of *Chlorella* in phosphate buffer. Nitrogen atmosphere. Effect of change to oxygen atmosphere.

strated on the curve of the type in Fig. 1 also. The height of the initial spike depended on the previous history of the sample. One-half hour of anaerobiosis produced a high spike. These induction effects were much reduced when the chemiluminescence was observed after only a short dark period of anaerobiosis. Treatment with oxygen during anaerobiosis in the dark, followed by careful flushing of the oxygen

before irradiation, caused the chemiluminescence to increase. The luminescence measured in the presence of carbon dioxide and nitrogen (no oxygen) was slightly greater than that measured with carbon dioxide and air present.

Figure 5 shows the effect of a change to pure oxygen following irradiation under pure nitrogen. Similarly, it was observed that the chemiluminescence was higher in the presence of ordinary tank nitrogen than in the presence of our specially purified gas.

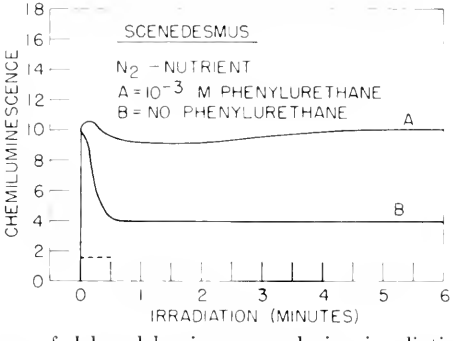

Fig. 6. Time course of delayed luminescence during irradiation of *Scenedesmus* in nutrient solution. Nitrogen atmosphere. Effect of *N*-phenylurethane.

The conclusion that the chemiluminescence is least intense when photosynthesis is proceeding with great facility is bolstered by studies made with various poisons, inhibitors, and narcotics. A curve made using *Scenedesmus* to which *N*-phenylurethane had been added is shown in Fig. 6.

The intensity of the chemiluminescence in the steady state is roughly linear with irradiation intensity under the conditions of our experiments. (We normally operated in the region of near saturating intensities.) This is to be contrasted with the finding of Arnold that the luminescence saturates at low intensities of irradiation. Whether one observes a saturation or a linear rise with increasing irradiation depends on the time delay between irradiation and measurement of the luminescence. In our experiments, the time elapsed is less than a millisecond, whereas in those of Strehler and Arnold it is of the order of tenths of seconds.

I shall not discuss these experimental results and observations in

detail. It is perhaps sufficient to quote from an abstract composed by Professor Franck. He prepared these remarks before his illness made it impossible for him to be here. Professor Franck wrote as follows:

"Our theory of the photochemical part of photosynthesis presupposes a main process of photosynthesis in which the energy of two absorption acts is utilized for the transfer of one hydrogen atom to the photosynthetic oxidant PGA and the simultaneous transfer of a hydroxyl to an enzyme. There is a minor process in which DPGA is reduced to -PGAH and phosphate whereby the energy of only one absorption act is used.* The early products of this process are the -PGAH radical and the chlorophyll radical which has lost one H-atom in ring V and is connected with two hydroxyls bound to C_9. The latter radical is a potential OH donor as long as the OH-carrying enzyme has not removed one of the hydroxyls. This reaction is slow because a relatively high heat of activation is needed. Thus the potential OH donor has a much longer lifetime than the corresponding products of the main process. Back reactions between the potential OH donor of the second process and a -PGAH radical will therefore occur relatively often even if enough active enzyme for the removal of OH is available. The particular back reaction assumed is a removal of the H which the PGA has gained in the forward process by a reaction with one of the hydroxyls of the chlorophyll radical. This back reaction results in the re-formation of PGA and of chlorophyll in the enol state. The energy released by the reaction is quite sufficient to excite the chlorophyll. Other factors very favorable for the occurrence of chemiluminescence are that the back reaction must occur in direct contact with chlorophyll and that most probably the enol chlorophyll has a higher fluorescence yield than the keto chlorophyll.

"Certainly back reactions will also occur between the two radicals PGAH- and HO-Enz- of process I, especially, if by addition of inhibitors, their lifetimes are prolonged. However, these back reactions have little chance to excite the chlorophyll because they will occur everywhere in the solution and not just in contact with chlorophyll. This interpretation can explain the differences of the chemiluminescence observed directly after irradiation and after a dark pause of two-tenths of a second. It is further in agreement with observations

* Compare addendum to "A Theory of the Photochemical Part of Photosynthesis" on page 142.

on the influence of inhibitors on the intensity of chemiluminescence and on the quenching influence of carbon dioxide."

A presentation of other data obtained in this study, as well as a theoretical consideration of all the phenomena of chemiluminescence, is in preparation.

Discussion

Rabinowitch: It should be reemphasized that you used pulsed light to irradiate your algae.

Arnold: We measured the chemiluminescence of Krall's barley and found spikes eight times higher than the steady state. This agrees with your observations for *Chlorella* in air containing 2% carbon dioxide.

Witt: Did you observe the high spikes when you resumed pulsed irradiation after having kept the *Chlorella* in the dark for a minute or so?

Brugger: No. I obtained the highest spikes with *Chlorella* samples which had been kept in the dark for an hour or so. If I stopped the irradiation after the steady state had been reached and allowed the *Chlorella* to remain in the dark for

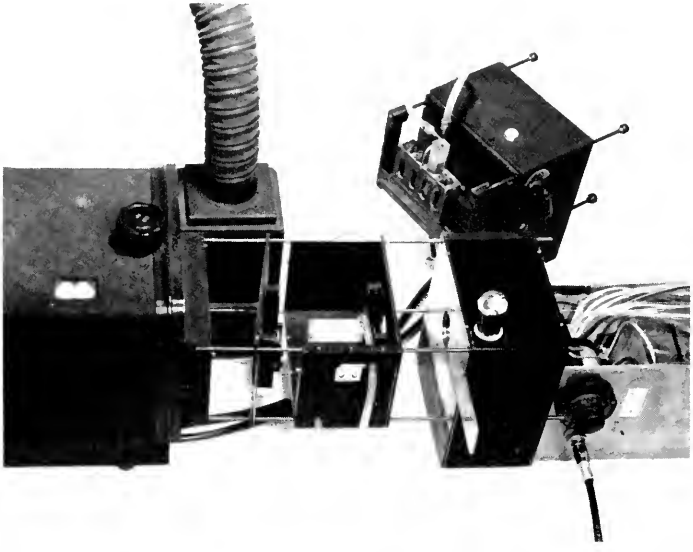

Fig. 7. Recording monochromatic photometer: (front, left to right) Beckman DU monochromator, cell and filter holder fitted with flow-type "Lucite" cell, housing for photomultiplier tube and battery power supply. Not shown: photosignal amplifier and recorder, device for irradiating algae, apparatus for circulating algal suspension through cell.

only a minute, only a very small spike was observed. The previous steady-state level was almost immediately reached. However, after 5 or 10 minutes in the dark, following an irradiation, the chemiluminescence spike was considerably higher.

Gaffron: It appears that the highest level of chemiluminescence you obtained by adding successive bursts of carbon dioxide to *Chlorella* in oxygen-free and carbon dioxide-free nitrogen is comparable to that which you found for *Chlorella* in pure oxygen. The steady-state level of chemiluminescence appeared to be highest in oxygen, lower in pure nitrogen, and lowest in the presence of carbon dioxide, with or without oxygen.

Brugger: This is correct.

Lumry: You told me that Dr. Frank Allen and you had studied changes in the absorption spectrum of *Chlorella* suspensions containing phenylurethane. Did you look at any other inhibitors for their effect on the spectrum? We have looked at a number of inhibitors which do affect the light reaction and have detected no changes in spectrum at all at high light intensities. We included agents which quench the fluorescence. I wonder whether there is any indication that these agents have a permanent absorptive effect on the chlorophyll.

Brugger: Dr. Allen and I built a recording monochromatic photometer by modifying a Beckman DU spectrophotometer. We constructed a special cell and cell holder and a detecting, amplifying and recording unit, consisting of a multiplier phototube, Leeds and Northrup DC microammeter, and Speedomax recorder. Our electronics were such that we bucked most of the photosignal and amplified only the residual. We were able to repeat Dr. Duysens' observations on changes in absorption. *Chlorella* in water containing 3×10^{-3} *M* phenylurethane appeared to show a slight shift to the red in the absorption spectrum. We believe that the complicating feature of the enhanced fluorescence due to the phenylurethane was eliminated by judicious use of filters. As there is a definitely tentative character to our results, I do not wish to discuss them at length

A Theory of the Photochemical Part of Photosynthesis

JAMES FRANCK,* *University of Chicago (Fels Fund), Chicago, Illinois*

The fluorescence yield of chlorophyll *a* in green plants is low and, very roughly speaking, constant over the range of illumination intensities from that which compensates respiration to the one which produces saturation. When secondary processes involving molecular oxygen are avoided, the fluorescence yield remains unaltered well into the region of saturation. This indicates that the processes principally responsible for the limitation of the fluorescence yield are those associated with the excitation of the chlorophyll molecules rather than with the utilization of the excitation energy for photochemical purposes. The transition of the excited chlorophyll molecule into the metastable triplet state is the principal cause of the low fluorescence yield. Since the energy of this state is not sufficient to transfer one hydrogen atom from water to the usual photosynthetic oxidant at a sufficiently great rate (from a donor capable after dehydrogenation to initiate a series of reactions leading to oxygen evolution), we conclude that the cooperation of two absorption acts is needed. For several reasons, we are convinced that phosphoglyceric acid itself is the primary H acceptor. The hydroxyl of the water will, according to our picture, be transferred to an enzyme involved in photosynthetic oxygen production. Since chlorophyll hydrates easily, we introduce the assumption that the oxygen on C_9 in the keto form of chlorophyll *a* will be hydrated. Then the steps by which an H atom is transferred to the PGA are:

1. Excitation of the chlorophyll molecule to its first excited singlet state.

2. Transition to the metastable triplet state.

3. Adsorption of PGA and of the enzyme which acts as OH acceptor by chlorophyll in its long-lived metastable state. This process occurs because the metastable state of chlorophyll has qualities similar to those of a bi-radical.

* As presented by J. E. Brugger.

4. Transition of the chlorophyll from its metastable triplet state to the first excited triplet state via the process of sensitized fluorescence. A molecule of chlorophyll in the first excited singlet state is degraded to the ground state, while another molecule in the lowest metastable state is raised to the first excited metastable state.

5. Utilization of the stored excitation energy for the transfer of the H atom bound to C_{10} to the PGA and of one of the hydroxyls bound to C_9 to the enzyme. The formation of a double bond between C_9 and C_{10} is part of this process and leaves the chlorophyll in the ground state in its enol form.

A number of enzymatic dark reactions follow the production of the two radicals. We enumerate: (*1*) The chlorophyll must revert to its hydrated keto form. (*2*) Several enzymatic reactions are connected with the oxygen evolution. (*3*) The PGAH radicals are, in this theory, supposed to dismute enzymatically into triose and PGA. Considerable energy will be released in this process and can be used for ATP production. The energy stored in ATP is supposed to be utilized in the Calvin cycle, by which the carbon dioxide acceptor, ribulose diphosphate, is made fro trimose.

Practically the same process is postulated for the main course of the reduction of Hill reagents. Since they are stronger oxidants, a second process can contribute to their reduction to some degree. The stronger oxidants, like quinone or oxygen, are quenchers of the chlorophyll fluorescence. Since the concentrations of the Hill reagents used in these experiments are low, the quenching impacts are not frequent enough to reduce the fluorescence intensity by more than 20% to 30%. The quenching is due to the utilization of the excitation energy for an H transfer to the oxidant. The energy available in one chlorophyll molecule in the singlet state is sufficient for this reaction. Thus only two steps occur in this process: excitation of the chlorophyll to the first excited singlet state and transfer of an H atom to the oxidant, for instance, to the quinone. The result will be the formation of a semiquinone and of a chlorophyll radical, which still retains both its hydroxyls. The energy needed to remove one of the hydroxyls from this radical is small because this removal permits the double bond between C_9 and C_{10} to close. However, a heat of activation is required for the transfer of one of the OH's to the enzyme. In photosynthesis, too, the second process may play a minor but significant role. The energy relations will permit a hydrogen transfer in one act

to diphosphoglyceric acid* if the energy stored in one of the phosphate bonds is utilized for the reaction. According to this theory, diphosphoglyceric acid is not the ordinary oxidant of photosynthesis, but it will contribute to photosynthesis when it is present in a high enough concentration as a respiratory intermediate. We believe that this is the case at illumination intensities below the compensation point and, as Dr. Brugger has reported, a corresponding quenching of the chlorophyll fluorescence has been found there (see page 113).

ADDENDUM

The theory outlined above has, in the meantime, been useful for a better understanding of certain other observations with photosynthesizing cells. On the other hand, two changes in the discussion of the "minor" process of photosynthesis and reduction of Hill reagents seem to be indicated:

1. This "one quantum process" for the transfer of an H atom from the chlorophyll in its first excited singlet state to the oxidant becomes, in effect, a two-quantum process above a certain low intensity of irradiation, since under those conditions the energy of a second quantum will be utilized for the transfer of one hydroxyl to the enzyme. Only at quite low intensities will a thermal fluctuation sufficient for the hydroxyl transfer occur before an excitation of the radical takes place (mostly by energy transfer, as in sensitized fluorescence). These deductions are based on observations of the chlorophyll fluorescence and chemiluminescence in living cells.

2. While reduction of PGA coupled with the transfer of one hydroxyl to the OH accepting enzyme undoubtedly needs the energy of two quanta per hydrogen transferred, indications are that PGA (and not only DPGA) can quench the chlorophyll fluorescence by utilizing the excitation energy for the transfer of the H atom on C_{10} to the photosynthetic oxidant. In that case, the heat of activation for the H transfer (and the corresponding loss of energy) must be considerably smaller than for the OH transfer. Furthermore, the bond between the hydrogen atom and C_{10} must be weaker than was anticipated by us. This hypothesis about the heat of activation is quite plausible. That the bond between the H and C_{10} is weaker than normal has been deduced quite early by Conant from chemical evidence.

* A precursor of PGA in the respiratory cycle.

Discussion

Gaffron: We have expressed regret that Dr. Franck could not be here. This, I feel, applies in particular to our discussions on the chemiluminescence data. What has been missing is a more thorough debate in biochemical terms on what Dr. Franck thinks is the most logical interpretation of Strehler's and Arnold's, as well as Brugger's, observations.

Dr. Franck's main contention is that there is no necessity to introduce a hydrogen transferring enzyme for the photochemical reduction of PGA (or whatever carboxylated substance becomes reduced). The task of an enzyme is to facilitate reactions which otherwise require too much activation energy. In the photochemical reaction we have an excess of energy to take care of activation.

Dr. Franck believes the majority of observations available to date indicate that a photochemical hydrogen transfer happens, indeed without a transferring agent, directly between the chlorophyll complex and an attached ultimate acceptor. Such a direct transfer could also be written in the form of an electron movement though, he contends, this entails more conceptual difficulties than is generally recognized. What the biochemist should consider carefully is whether it is wrong, for experimental reasons, to assume that a reducible intermediate, say of the Calvin-Benson cycle, is directly attached to the chlorophyll complex. By means of the one or two quantum process (which one is here irrelevant) the metabolic intermediate receives one hydrogen and becomes a radical. Then it has to wait for the opportunity to get a second hydrogen. During this time it can react back and produce luminescence. Franck belongs among those scientists who believe that a theory becomes respectable only after it has been worked out and revised to a point where no experimental contradictions are left that are fairly obvious. I remind you of Dr. Brugger's curve showing the chemiluminescence of *Chlorella* in nitrogen during steady-state illumination. The luminescence is relatively low. Then one puts in a little carbon dioxide (see Fig. 4 on page 137). Since carbon dioxide causes photosynthesis to start, energy is drained away and one gets a depression in the luminescence immediately followed by an increase. Upon another addition of a little carbon dioxide, the luminescence again goes down and then up. It can be pushed up in steps by adding small amounts of carbon dioxide to the maximum the luminescence can reach under any steady-state conditions. There is a staircase effect due to short-lasting additions of carbon dioxide. This is typical for one of those observations which require an explanation consistent with the biochemistry and the physics of the process before one can say the matter is understood. Higher luminescence means a higher concentration of photochemically produced radicals. Here they seem to increase in proportion to the amounts of CO_2-dependent intermediates, whenever the latter are deprived of the opportunity to complete the cycle.

Bassham: I think we can explain this easily in terms of intermediate-reduced enzymes; and, secondly, I would point out that as far as photochemical reduction of PGA is concerned, we can reduce PGA in the dark following preillumination.

Gaffron: In this case PGA is formed by carboxylation and some of it is certainly reduced in the dark. Does this prove it is exactly the same reaction which goes on in the light?

Benson: But you can do it in the dark just as fast as in the light.

Bassham: Why call on a second way when you already have one which works?

Gaffron: Because many other data do not quite fit this rather plausible assumption. I am a biochemist, too. My way of thinking is like yours, and it is only from contact with Prof. Franck that I am learning to distrust this simple way of explaining by analogy. From the biochemical viewpoint, we have here an obvious explanation of how the light can be used very efficiently, but the data of the photochemists unfortunately do not quite jibe with the idea of a primary intermediate hydrogen acceptor which in turn reduces everything else. Dr. Franck chooses to worry about the existent difficulties. Some of us may believe it is unnecessary to worry. But the fact remains that there are some reliable data which are difficult to explain unless one assumes that at least one component of the Benson-Calvin cycle sticks very close to the chlorophyll itself.

Part III

THE POSSIBLE ROLE OF CYTOCHROMES

Hematin Compounds in the Metabolism of Photosynthetic Tissues

MARTIN D. KAMEN, *Edward Mallinckrodt Institute of Radiology, Washington Medical School, St. Louis, Missouri*

Two salient facts, established only recently, provide the basis for this discussion. One is that all photosynthetic tissues contain hematin compounds. The second concerns the effect of the light energy utilized in photosynthesis. This energy sets up a steady state in which the major change relative to the dark steady state is a shift in the direction of greater oxidation of one or more hematin compounds.

In the case of green plants and algae, it is not difficult to limit the data to be considered to those known to be unique to photosynthetic tissue. In these organisms, the sites of photometabolism are well-defined subcellular particles ("chloroplasts") about which a large body of knowledge is steadily accumulating. Much less well defined are the corresponding entities in the bacteria. This uncertainty is not serious in the case of the obligate photoanaerobes, because in these systems growth is possible only by photosynthesis under strictly anaerobic conditions. In the facultative bacteria, there is evidence that cell particles much like chloroplasts exist in at least two species, *Rhodospirillum rubrum* and *Rhodopseudomonas spheroides* (31,33,37).

There is a great deal of significance in the fact that hematin compounds are found both in green plant chloroplasts and in large amounts in all varieties of the photosynthetic bacteria. This significance lies in the fact that, whereas the green plants, algae, and the photosynthetic bacteria cover practically the whole range of living metabolic patterns, they share no property except that of using light energy for growth. Furthermore, until now only two classes of compounds have been found in all photosynthetic systems. These are the photoactive pigment complexes—the chlorophylls, carotenoids, etc.—and the hematin compounds.

The significance of the observation that absorption of light energy results in oxidation of component hematins lies in the apparent magnitude of these changes, in both amount and duration, which

dwarf changes in other components expected to participate in energy transfer during the early phase of photosynthesis.

In elaboration of these remarks this report will be concerned, first, with the data relating to distribution and nature of hematin compounds (21) in photosynthetic tissue, and, second, with the observations which have been made on the steady state of oxidation of cell components during photosynthesis.

OCCURRENCE AND NATURE OF HEMATIN COMPOUNDS IN GREEN PLANT CHLOROPLASTS

Two hematin compounds appear to be unique to the photosynthetic apparatus in green plants. One of these, cytochrome *f*, was the first chloroplast hematin compound to be detected (30). Its isolation and properties have been described exhaustively by Hill and his collaborators (8,15,16).

Cytochrome *f* is widely distributed in the higher plants and algae. It appears to be an integral part of the chloroplast structure inasmuch as it cannot be separated from the chloroplast unless organic solvents are used to split off lipid. Cytochrome *f* is estimated to account for at least one-third of the hematin of the chloroplast and is present in the ratio of 1 mole for approximately 400 moles chlorophyll (8,16,24). It has been possible to obtain a preparation purified some 300-fold over the state in which cytochrome *f* is initially extracted from the leaf, using ammoniacal ethanol. The yield of purified material is about 13% of the hematin originally present in the crude extract.

Cytochrome *f* is classified as a "modified" cytochrome of the *c* type because of its spectrochemical characteristics, the spectra of the hemochromogens derived from it by reaction with alkaline cyanide or pyridine, and its relatively high potential. However, it will not function as a substrate for the classical cytochrome *c* oxidase. In fact, no system spectrochemically analogous to cytochrome *a* or a_3 appears to be present in the chloroplast, although it exists in the cytoplasm outside the chloroplast along with normal cytochrome *c*. Oxidase activity responding to respiratory inhibitors has been reported in chloroplasts (29) but such activity is not proof for the presence of cytochrome *a*. Cytochrome *f* will couple with cytochrome *c* nonenzymatically so that it can be oxidized slowly when incubated

in air with cytochrome *c* oxidase and catalytic amounts of cytochrome *c*.

The other chloroplast hematin compound is a "*b*" type cytochrome, called b_6 by Hill (14). This hematin compound appears to be associated with the chloroplasts of a variety of plants. Like cytochrome *f*, it is bound in the chloroplast structure firmly enough to withstand extraction by aqueous solvents.

Cytochrome *f* and cytochrome b_6 cover a range of redox potential which is more than half of the total required to span the difference between the hydrogen and oxygen electrodes at physiological pH (14). Thus, these two compounds provide the chloroplast with an electron transfer system which, in principle, could carry out oxidations in either direction over a large fraction of the physiological range.

HEMATIN COMPOUNDS OF FACULTATIVE PHOTOSYNTHETIC BACTERIA

The nonsulfur purple bacteria are classified at present into two genera, *Rhodospirillum* and *Rhodopseudomonas* (35). Representative species contain large amounts of soluble hematin compounds which can be described as modified "*c*" cytochromes (19). Like cytochrome *f*, all these heme proteins show high positive redox potentials. Again like cytochrome *f*, they are not substrates for a cytochrome *a* type oxidase, and they can be coupled nonenzymatically with cytochrome *c*.

The heme protein from *R. rubrum* has been studied most inten-

TABLE I. Some Properties of *R. rubrum*-Cytochrome *c* Compared with Mammalian Cytochrome *c* (38)

	R. rubrum-cytochrome *c*	Mammalian cytochrome *c*
Classification	Modified *c*	Standard *c*
Stability	Resistant to extremes of heat and acidity	Resistant to extremes of heat and acidity
Autoxidation	None	None
Reaction with CO on reduction	None	None
Isoelectric point (pH)	Approximately 7	10
Electrophoretic mobility pH 7 (cm.^{2}/volt-sec.)	Cathodic, 3.1×10^{-5}	Cathodic, 8.2×10^{-5}
Absorption on IRC-50, pH 7	None	Strongly absorbed

sively (10,19,36). Its properties, which are typical of many "*c*" cytochromes in photosynthetic organisms, are exhibited in Table I.

The facultative bacteria yield these proteins readily upon treatment with warm trichloroacetic acid as in the Keilin-Hartree procedure, and can be purified in the same manner as mammalian cytochrome *c* (19,38). However, they are noticeably more labile than their mammalian analogs and can be obtained in better yield with less denaturation by use of less drastic methods (19,20). In *R. rubrum* and *R. spheroides*, most of the cytochrome "*c*" appears to be loosely bound and extractable. There also is a fraction which is tightly bound to the particles and resists extraction with aqueous systems just as do the chloroplast hematins.

There is some evidence that cytochromes of the "*b*" type are present in the chromatophores of the facultative bacteria. However, only one such hematin compound has been characterized (38).

A remarkable new type of hematin compound, as yet unclassified, has been observed which is distributed generally in the facultative bacteria (19,38). Its properties are listed in Table II. This compound displays the spectroscopic properties of a compound of the myoglobin type and shows, as well, an ability to form a CO complex in the reduced form. Nonetheless, it yields hemochromogens with spectra like those formed from a typical cytochrome *c*. The redox potential lies in the region normally attributed to hemoglobins or cytochrome *b*. It is clear that this hematin compound cannot be classified (at least, so far) in any of the common categories of the iron porphyrin proteins.

The enzymatic properties of this compound are also remarkable

TABLE II. Some Properties of *R. rubrum* Yellow Pigment (38)

Classification	Hybrid
Stability	Thermostable, slowly denatured at low pH
Autoxidation	Rapid
Reaction with CO in reduced form	Forms CO complex
Absorption maxima (reduced)	550, 423 mμ (no beta band)
Absorption maxima (oxidized)	640, 490–500, 393 mμ
E_0' (pH 7)	~0.1 to +0.15 mv
Reduced pyridine hemochromogen	Spectroscopically identical with that obtained from mammalian cytochrome *c*
Reduced cyanide hemochromogen	Spectroscopically identical with that obtained from mammalian cytochrome *c*

(38). It can act as a substrate for either the bacterial or mammalian cytochrome *c* reductase (25). Since it is rapidly autoxidizable and forms a CO compound in the reduced state it could function as a terminal respiratory oxidase. Another interesting property of this compound is its ability to reduce the bacterial cytochrome *c* nonenzymatically. In this respect it behaves in a manner reminiscent of mammalian cytochrome *c* with the bacterial cytochrome *c* or cytochrome *f*. In other words, it provides a sort of oxidase for the cytochrome *c* of the bacteria as well as for the low potential electron donors present in the oxidative system.

There is no satisfactory name for this compound at present. It has been called variously "pseudohemoglobin," "yellow pigment," and "CO-binding pigment." The difficulty of naming and classifying it can be resolved only by precise determination of its functions in bacterial metabolism.

HEMATIN COMPOUNDS OF STRICTLY ANAEROBIC PHOTOSYNTHETIC BACTERIA

The green sulfur bacteria and the purple sulfur bacteria make up two divisions of the group of photosynthetic bacteria (34). They are marked off from the other division, the nonsulfur purple bacteria, because they can grow only as photoanaerobes. In the absence of light, they cannot use energy derived aerobically or anaerobically for growth. Thus, they provide a unique opportunity for the study of the function of hematin compounds in systems uncomplicated by pathways for utilization of energy other than that available in photosynthesis.

In the green bacteria, only one genus—*Chlorobium*—is recognized (22). The few species known are differentiated from each other by characteristic substrate requirements for growth. Three hematin compounds are known. The first to be isolated in cell-free extracts is an iron porphyrin protein from *C. limicola* (17). Two others have been detected in *C. thiosulfaticum* (13). The properties of *C. cytochrome*-554 (1) and the *C. limicola* pigment are summarized in Table III. It is seen that these pigments while resembling a cytochrome of the "*c*" type show redox potentials more characteristic of cytochromes of the "*b*" type.

One hematin compound, isolated and purified from the purple sulfur bacterium, *Chromatium*, strain D, has been studied (26). Purifi-

TABLE III. Cytochrome Components of Green Sulfur Bacteria (13,17)

C. limicola, cytochrome-553; see (17)	
Absorption maxima (reduced)	553, 520, 415 mμ
Reaction with CO in reduced form	None
Autoxidation	Slow
C. thiosulfaticum, cytochrome-554 (1); see (13)	
Absorption maxima (reduced)	554, 523, 417 mμ
Absorption maxima (NO-compound-oxidized)	573, 537 mμ
Reaction with CO in reduced form	None
Autoxidation	Slow
Fe content	0.37%
Hematin content	3.1%
E_0' (pH 7)	$\sim +0.160$ volt
Concentration in cells	$\sim 0.1\%$ dry weight

cation beyond that achieved by exhaustive electrophoresis of crude ammonium sulfate fractions has not been obtained.

The properties of the purest preparation obtained are shown in Table IV. At least two protein components are present. One of these binds a heme group similar to that found in mammalian cytochrome *c*. There is nonheme iron apparently present in some as yet unspecified form. While the compound is slowly autoxidizable and exhibits spectroscopic behavior reminiscent of cytochrome *c*, it has a low redox potential. Thus, it, too, falls into no recognized single class of

TABLE IV. Properties of Chromatium Cytochrome (26)

Absorption maxima (mμ)	
Ferrocytochrome	552, 525, 418 (shoulder at 423)
Ferricytochrome	—, —, 406
Reduced cyanide hemochromogen	553, 527, 419
Reduced pyridine hemochromogen	551, 519, 412
Porphyrin in ether	632, 575, 532, 503, 375
Ratios of ferrocytochrome maxima	
270/552	8.57
418/552	9.8
552/525	1.16
Anodic mobility, pH 6.0	5.9×10^{-5} cm.²/volt-sec.
" " , pH 7.8	6.26×10^{-5} cm.²/volt-sec.
	8.43×10^{-5} cm.²/volt-sec.
Fe content, % protein	0.12
Ratio; Fe/Heme	2.0 to 2.7
E'_0 (pH 7)	−0.04 volt

iron porphyrin protein but is a hybrid compound like the cytochrome of the green bacteria and the yellow pigment of the facultative bacteria.

No enzymic function has been found for this compound, just as in the case of cytochrome *f*. Cytochrome *c* reductase fails to activate the *Chromatium* pigment, although extracts of *Chromatium* contain reductase activity when cytochrome *c* is present as a substrate (27).

It is apparent that the photochemical apparatus in photosynthetic systems, regardless of overall metabolic pattern, contains hematin compounds. Not only do these hematin compounds appear to be unique but they also do not occur outside the photochemical apparatus under conditions where there is no close coupling between normal respiration and photosynthesis.

THE FUNCTION OF HEMATIN COMPOUNDS IN PHOTOSYNTHESIS

In green plant photosynthesis, the net result of the photochemical act is the transport of four electrons against a potential drop of 1.2 volts, which is the difference between the potentials of the hydrogen and oxygen electrodes at physiological pH. If the unitary theory of photosynthesis is adhered to (34), a similar process may be supposed to occur in bacterial photosynthesis, although no oxygen evolution concomitant with reduction at the level of the hydrogen electrode can be demonstrated.

In 1939, Hill proposed (see 15) that the initial phase of the photochemical process did not push electrons all the way from the oxygen potential to the hydrogen potential, but only part way. A back oxidation was invoked to provide the additional energy storage. He suggested that the substrate for the back oxidation was a part of the reduced material formed in the initial photochemical act, and the H acceptor was a part of the oxygen liberated by the partial movement of electrons away from the potential of oxygen. He also raised the possibility that the transport system for this movement of electrons consisted of respiratory pigments similar to, but not identical with, those normal to respiratory systems. It was inferred that this photorespiratory system was separated in the cell, spatially or otherwise, so that it could function in close coupling with the forward reaction of light absorption and act independently of normal respiration. At the time of these proposals, no basis existed for the notion of chloroplast respiratory pigments. As we have seen, however,

there is now considerable experimental backing for the existence of such entities.

The notion of a back oxidation has intrigued many writers and has appeared in one form or another too often to recount here. (See, for example, 2,3,23,39.) Davenport and Hill (8) have elaborated the concept of a back oxidation coupled to the photochemical process in terms of the hematin compound, cytochrome *f*, and some other oxidizing agent which is reduced in the photochemical act. They point out that the potential for cytochrome *f* lies at a point more negative than that of the oxygen electrode by an amount which, for the movement of four electrons, is precisely equivalent to the energy of one quantum in the characteristic red emission spectrum of chlorophyll. The next drop in potential brings one to the region at the limit of the Hill chloroplast reaction and to the potential of cytochrome b_6. Here, again, the potential difference results in an energy change for four electrons corresponding to the energy in one quantum of red light.

Gaffron and Rosenberg (12) have summarized the experimental findings which indicate that no back oxidation occurs involving molecular oxygen as such. However, *preoccupation with oxygen as the H acceptor in such a process is not requisite to the Hill postulate.* The oxidizing system generated in the light can be a substance other than oxygen, such as a complex organic free radical which produces oxygen irreversibly and partly back reacts in the manner suggested by Hill.

It is fortunate that techniques now exist for making a start in determining the state of cell constituents in intact cells during metabolism. These are dynamic spectrophotometric methods, which have been employed by Duysens (9), Lundegårdh (24), and others, and have reached a notable degree of development in the hands of Britton Chance and his co-workers (4–7).

Duysens first observed that, in intact cell suspensions of *R. rubrum*, there were changes in optical density brought about by a transition from a steady state in the dark to a steady state in the light. These changes coincided with the difference spectrum between oxidized and reduced forms of a cytochrome type compound or compounds. Vernon and Kamen had shown about the same time (37) that the *R. rubrum* cytochrome *c* could be photochemically oxidized by air in an enzymic reaction, and that compounds at potentials more negative than

that for the cytochrome component were not reactive (37). Shortly, thereafter, Chance and Smith (6,7) conducted a more exhaustive spectrophotometric analysis of the action spectra exhibited by *R. rubrum* cell suspensions under a variety of experimental conditions. In agreement with the other workers, they found that the net effect of illumination was an overall oxidation of one or more hematin compounds. In this facultative bacterium, which displays competition between dark aerobic oxygen uptake and light anaerobic metabolism, the effect of light under anaerobic conditions was qualitatively the same as that in the dark when oxygen was admitted.

Chance and Smith (6) have proposed a scheme in which the hematin compounds (*R. rubrum* cytochrome *c* and yellow pigment) act as a bridge for electron transfer between the products of photolysis and the systems concerned with reduction of CO_2 and other substrates. Their basic assumption is that the hematin chain which reacts with oxygen can also react with the products of photolysis. They place the yellow pigment at the end of the respiratory chain. The redox potential in the isolated form appears to be negative by more than 100 mv. compared to the cytochrome *c* component (38). This fact would seem to disagree with the notion of the yellow pigment as a terminal oxidase. However, it has not been established that the yellow pigment has not undergone some modification during the procedures employed for extraction.

Kamen and Vernon (18) have shown that, in *R. rubrum*, the reductase activity far exceeds the oxidase activity, using bacterial cytochrome *c* or mammalian cytochrome *c* as substrate. Hence the cytochrome *c* should be largely in the reduced state in the dark under aerobic conditions, as well as anaerobic conditions. Chance and Smith (6) find that addition of phenyl mercuriacetate causes a rapid oxidation of the cytochrome *c*. This is in agreement with the known inhibition of reductase activity by this agent. In the absence of this inhibitor, they suppose that the photooxidant can react with the yellow pigment and thus pull electrons away from the cytochrome in a manner analogous to that observed by Vernon and Kamen for the coupled oxidation of cytochrome *c* by air in the presence of yellow pigment (38).

These latter workers have also shown that the photoenzymic oxidation of cytochrome *c* is not inhibited by cyanide as is the oxidase of the aerobic system in the dark (37). Similarly, Chance and Smith

(6) note that the light-induced oxidation is not affected by cyanide, and in addition is insensitive to CO. The isolated pigment has been described previously as forming a CO-complex, but apparently this compound may not be effective in preventing light oxidation because of the well-known dissociation of such heme-CO compounds by light. Both groups of workers postulate a competition between the photolytic oxidant and oxygen, with the site of the competition in the hematin chain. Chance and Smith (6) propose that the shift to the oxidized state is the result of the preferential attack of the oxidase by the photooxidant.

Kamen and Vernon (18) have observed that the photooxidation in air catalyzed by their photooxidase proceeds at a rate at light saturation (which is attained at rather low light intensity) some five to ten times faster than the rate of the dark oxidation under the same conditions. They argue by analogy from this observation that competition occurs at the cytochrome *c* level. In this scheme, the bacterial cytochrome would act as the substrate for the two oxidative systems and the light oxidase would compete successfully for the cytochrome *c* during illumination. The shift from reduced to oxidized cytochrome, on their viewpoint, results from the fact that while the dark oxidase cannot keep up with the reductase in the absence of light, the increased rate of oxidation possible with the light oxidase favors a greater degree of oxidation in the light.

The complexities which arise in the interpretation of shifts in oxidation states in faculative tissues should be obviated by similar experiments in tissues of strictly photoanaerobic character. A start has been made by Olsen and Chance with *Chromatium* (28). Their preliminary findings again indicate that practically the entire action spectrum found can be accounted for in terms of photooxidation of the hematin system as isolated and characterized by Newton and Kamen, described above (26). This finding, even though preliminary, provides strong evidence that the hematin system of photosynthetic tissue is coupled to the photochemical act, because no other function for the hematin system is available in such a tissue. The bacterium cannot use a chemosynthetic oxidation apart from photometabolism to any useful purpose, and in fact, is inactivated and finally rendered nonviable by air.

A number of observations on chloroplasts and intact green plant tissues have been reported which parallel those recorded for the bac-

teria in addition to the observations reported previously in this symposium. Lundegårdh has claimed that, in *Chlorella*, illumination causes an oxidation of cytochrome *f* and a barely detectable but significant reduction of some *b* cytochrome (24). He has suggested a scheme similar in many respects to that provided by Hill, in which electron flow down the respiratory chain in the dark is reversed and run backward through a "special cytochrome system in which cytochrome *f* is one of the links" (24). Recently, Hill has remarked (14) that in leaves of certain "golden" varieties of plants a sharp band corresponding to reduced cytochrome b_6 appears on illumination *in vivo*, which can only be obtained from the chloroplasts isolated from the same sources by treatment with hydrosulfite. There is also an indication that, at the same time the *b* component is reduced, the *f* component is oxidized. Here, as in the bacteria, no components other than the hematin compounds in photosynthetic tissues show changes in oxidation state of this magnitude upon illumination. It is evident that this phase of research is in its infancy but like most infants it can be regarded with optimism for the future.

The acceptance of the notion that a back oxidation involving a chain of hematin compounds occurs closely coupled with the photochemical act would normally invite speculation about a subsequent or concomitant phosphorylation. Such a phosphorylation would be analogous to the coupling of phosphorylation and oxidation in normal respiration. However, no speculation is required, as experimental data are at hand which appear to indicate that the photochemical act can be coupled to phosphorylation under conditions where a normal respiratory oxidation cannot occur (i.e., under strictly anaerobic conditions). Frenkel (11), working with chromatophores from *R. rubrum*, has demonstrated that light under strictly anaerobic conditions induces a disappearance of inorganic phosphate in the presence of ADP which can be accounted for as newly formed ATP.

In well-washed chromatophores this light-dependent phosphorylation is not accompanied by appreciable dark phosphorylation. It is insensitive to the usual respiratory inhibitors and does not require oxygen. In this respect, the light phosphorylation parallels the photooxidation of cytochrome *c* by the same system (37). It is of interest that 2,6-dichlorophenol-indophenol which can act as a substrate in the photooxidation in air (37) suppresses the light anaerobic phosphorylation (11). Similar phenomena are noted in chloroplast sus-

pensions (1) and also in the chromatophores derived from the strict anaerobe, *Chromatium* (27). Thus, it appears that in both the plant and bacterial systems, sufficient separation of the photolytic products can be achieved in cell-free extracts to obtain a back oxidation in which useful biochemical energy can be stored.

In this connection, it is of interest to note that Wessels (40) has suggested a scheme for participation of hematin compounds in which phosphorylation is the end result of the light process. He suggests that vitamin K or a compound analogous to it is the natural Hill reagent in the chloroplast. According to his scheme, partial reoxidation of photochemically reduced vitamin K by cytochrome *c* (or cytochrome *f*) generates a high-energy phosphate bond(s) which may cooperate in the reduction of DPN (possibly proceeding via diaphorase). The oxidative phosphorylation of the reduced vitamin K by the cytochrome produces a tautomeric form of vitamin K in the phosphorylated state. The splitting of this compound could result in the formation of the more stable para-quinone structure of the vitamin, thus making the phosphate bond in this tautomeric structure energy-rich and available for conversion of ADP to ATP. Newton and Kamen have attempted to test this hypothesis of the involvement of vitamin K in the anaerobic system from *Chromatium*. The results were negative (27). Menadione, an analog of vitamin K, appears to be involved in the anaerobic phosphorylation by chloroplasts, however (1). It should be noted that although chloroplasts contain large amounts of vitamin K, there appears to be no appreciable amount of this vitamin in the anaerobic photosynthetic bacterium, *Chromatium* (27).

In all of this discussion there is a hint that the "splitting of water" as such may not be involved in the initial light reaction, but can occur instead as a result of the transfer and storage of energy during dark back oxidations of the type which have been postulated. In other words, single quantum events are assumed to mediate the production of ATP and other high-energy compounds. The evolution of oxygen is imagined to occur as a result of the gradual accumulation of a store of oxidized material. In the case of the bacteria, the oxidants are removed by reaction with exogenous H donors.

It is necessary in this connection to bring forward one more bit of speculation. The hematin compounds resemble chlorophyll and the other magnesium porphyrins closely enough so that direct excitation

of the hematin compound by inductive resonance can occur. The physical disposition of the cytochrome and other hematin components of the chloroplast is not known, but from the studies of various investigators (see, for instance, ref. 41) it appears that the chloroplasts consist of laminae in which fatty layers alternate with the aqueous phase. In the interface, the chlorophyll molecules lie close-packed on end, oriented so that the phytol chain dips into the lipid phase and the porphyrin-polar end binds to a protein in the aqueous phase. The hematin compounds would be expected to bind to protein in the aqueous phase, perhaps to the same protein as that which holds the chlorophyll. Thus there could be a bridge of hematin compounds connecting the chlorophyll-lipoprotein with the systems directly contiguous to the chloroplast which are involved in the secondary processes of CO_2 reduction, etc. Light energy absorbed by a given chlorophyll molecule would be likely to migrate through the interface until it found a cluster of hematin compounds, whereupon the whole energy of the quantum would become available to the hematin compounds. It is not difficult to imagine that absorption of the relatively large energy equivalent to one quantum would excite the heme protein to a state in which a dismutation reaction resulted. The result would be the production of partially dissociated ferroheme with one or more free Fe valences which could reduce H acceptors such as DPN at or close to the potential of the hydrogen electrode. After reduction, the ferriheme could "relax" into its original bound state with an "electron deficient region" left in the hydration envelope of the hematin compound. The continuation of this process would accumulate electron-deficient compounds in the phase surrounding the protein which could act as oxidants for back reactions and also as precursors of free oxygen. A fragment of such a mechanism is part of a recent suggestion by Warburg invoking a "photo-dissociation" of a heavy-metal binding compound coupled with a back oxidation of the dissociation products (39).

Acknowledgment. In conclusion, it is a pleasure to acknowledge the continued support of the C. F. Kettering Foundation, which in large part has made possible many of the studies which provide the framework for this report.

Discussion

Arnon: Did you get cytochrome *c* from the whole cells or from particles isolated from the cell?

Kamen: First, from the whole cell by sonic treatment. In this treatment, the supernatant becomes pink and you get most of your cytochrome that way. There is, however, always a certain amount of cytochrome left in the particles, which requires further treatment to get it out, and there is always some that does not come out at all.

Arnon: My question implied: what is the evidence for the statement that, in these bacteria, most of the cytochrome is in the particles?

Kamen: I did not say that most of it is in the particles. I said that in this bacterium most of it is easily extractable.

Arnon: Is that true of all photosynthetic bacteria or just of certain kinds?

Kamen: Only for these two we know about.

Lucile Smith: If you prepare the particles carefully, you can find all the cytochromes in them. It is only when you prepare them with the sonic vibrator that you get the cytochromes in solution.

Kamen: There is an enormous amount of cytochromes in these organisms by comparison with normal aerobic tissue. The amount is considerable even by comparison with live mitochondria.

[See also Discussion following paper by Albert R. Krall, pp. 320–325.]

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Investigations in the Photosynthetic Mechanism of Purple Bacteria by Means of Sensitive Absorption Spectrophotometry

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METHODS AND MATERIALS

Apparatus. The apparatus used is described in another paper in this volume.

Bacteria-Rhodospirillum rubrum strain 1 was obtained through the courtesy of Dr. C. B. van Niel, strain 4 from the Biophysical Research Group, Utrecht. Both strains were grown for 1 or 2 days in incandescent light, in stoppered test tubes completely filled with 1% Difco bactopeptone, strain 4 with the addition of $^1/_2$% sodium chloride. All experiments were done at room temperature: 18 to 24°C. An aqueous extract of the bacteria was prepared in the homogenizer according to French (*cf.* Milner *et al.* (1)).

The changes in absorption were measured in a 1-cm. Beckman cell; the optical densities of the bacterial suspensions at 880 mμ minus the optical densities at 960 mμ (to correct for scattering) were about 1.0 as measured with a Beckman DU spectrophotometer. These wavelengths were selected because the infrared maximum of bacteriochlorophyll in intact cells is located at 880 mμ, while the intrinsic absorption of the extracts is negligible at 960 mμ.

CHANGE IN ABSORPTION AT ONE WAVELENGTH

The time course of the changes in absorption appeared to depend upon the suspension medium and the intensity of the exciting light. Figure 1 shows the changes in optical density of a suspension of *Rhodospirillum rubrum* strain 4 at 430 mμ in anaerobic peptone. For intensities of the order of magnitude at which saturation of photosynthesis just occurs, the absorption decreases upon illumination

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(graphs I *a*, II *a*) until a steady-state value is reached within a minute; upon darkening the change is reversed (I *e* and II *e*). At very high intensities more complicated curves were observed (graphs III and IV). A decrease in absorption *a* was followed by an increase *b*, which in its turn was followed by a decrease *c*. Upon darkening a decrease *d* occurred followed by an increase *e*.

If the light was left on, the decrease *c* went on for minutes. In the dark this decrease was slowly reversed. The much faster changes *a*, *b*, *d* and the fast part of *e* took place also after long illumination had produced an appreciable, not yet reversed, "bleaching" *c*.

It seems that the changes *a* and the fast part of *e* of graphs III and IV are caused by the same pigment as the changes *a* and *e* in graphs I and II; *b* and *d* are presumably caused by a different pigment. Since the change *c* is slow, it is probably not caused by a photosynthetic catalyst, and, since the other changes seem to take place independently of *c*, it may be left out of consideration in the discussion of the changes *a*, *b*, *d*, and the fast part of *e*.

The bottom part of Fig. 1 shows that at 530 mμ changes corresponding to *a* and *e* are small (graph V) and that the changes *b* and *d* are pronounced (graph VI). It should not be concluded, however, that at 530 mμ the changes *b* and *d* are greater than at 430 mμ, since at 430 mμ these changes are counteracted by *a* and *e*.

DIFFERENCE SPECTRA

Under most experimental conditions the time course of the change in absorption is a monotonously increasing or decreasing function of the time, which approaches a steady-state value. Examples of such changes are graphs I and II of Fig. 1. If this steady-state value (at a certain constant exciting intensity) is plotted as a function of the wavelength of the measuring light, a "difference" spectrum is obtained.

The shapes of the difference spectra of *Rhodospirillum* appeared to depend on the suspension medium.

Three different spectra are represented:

1. The difference spectrum in anaerobic peptone (top curve of Fig. 2).

2. That in aerobic distilled water (bottom curve of Fig. 2).

3. That in anaerobic distilled water (Fig. 3).

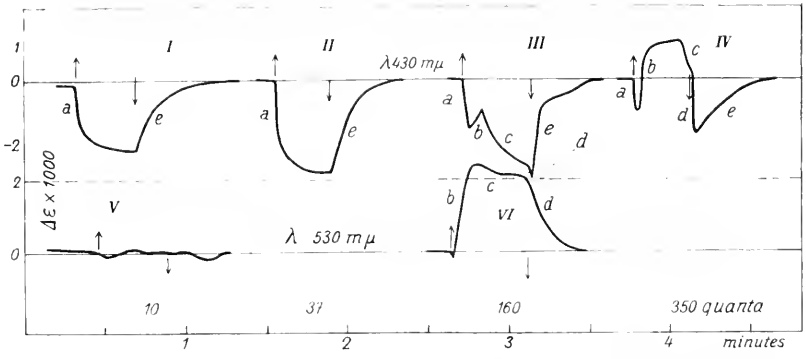

Fig. 1. Changes in optical density upon illumination of a *Rhodospirillum* suspension in anaerobic peptone at 430 (top figure) and 530 mμ (bottom figure). Onset of illumination is indicated by an upward pointing arrow and darkening by a downward pointing arrow. The numbers written at the bottom of the figure are the approximate number of quanta absorbed per minute per bacteriochlorophyll molecule.

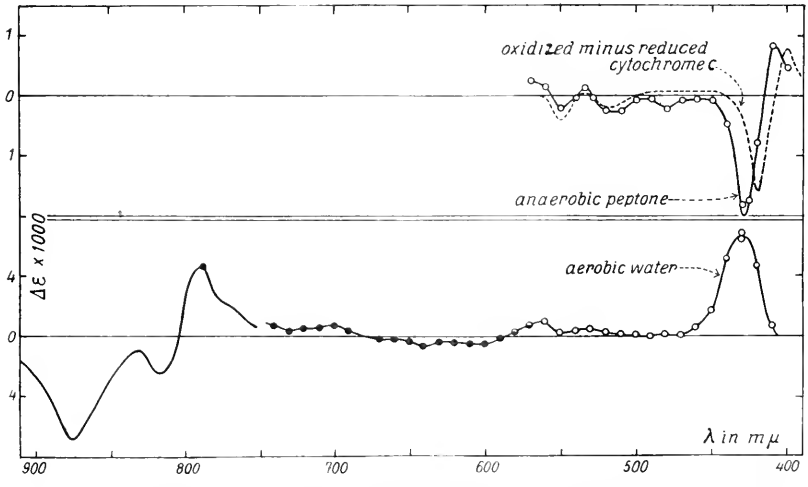

Fig. 2. Difference spectra of *Rhodospirillum* strain 1 in anaerobic peptone (top figure) and in aerobic distilled water (bottom figure). The left part in aerobic water is for strain 4, the right part for strain 1, and the middle part for an aqueous extract of strain 1; two parts were multiplied by factors to get an approximately continuous connection between the curves.

The difference spectrum in anaerobic peptone is similar to (albeit not identical with) the difference of the absorption spectra of oxidized and reduced cytochrome *c*. This indicates that upon illumination a cytochrome becomes more oxidized. Until its identity has been

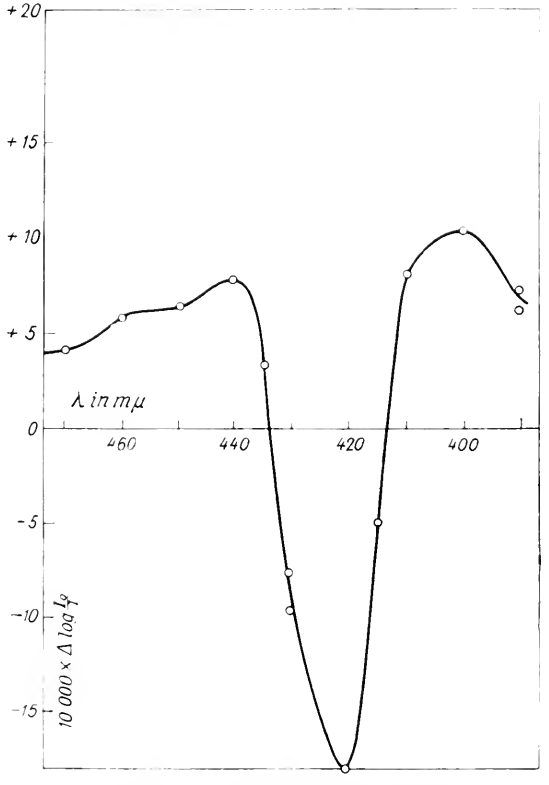

Fig. 3. Difference spectrum of *Rhodospirillum rubrum*, left 1 day in water after flushing with hydrogen ("anaerobic water"). Exciting light was a band at 540 mμ with intensity of 1.2×10^4 ergs/(cm.^{2} sec.).

established, we shall call this cytochrome "cytochrome 428" after the wavelength of the maximum in its difference spectrum.

In aerobic distilled water there is a positive maximum (increase in absorption) at 432 mμ. This is not caused by reduction of cytochrome 428 or of *Rhodospirillum* cytochrome *c* (3), since the band is too broad and occurs at another wavelength. In the infrared, there is a

decrease at 880 mμ and an increase at 790 mμ. The infrared part of the spectrum is probably caused by a change in bacteriochlorophyll (2). Since this type of difference spectrum occurs in the presence of oxygen or oxidizing conditions and disappears under reducing conditions (*cf.* 4), the change may be caused by an oxidation of bacterio chlorophyll. If a small part of the bacteriochlorophyll takes part in this reaction, then the difference spectrum indicates that the 880-mμ

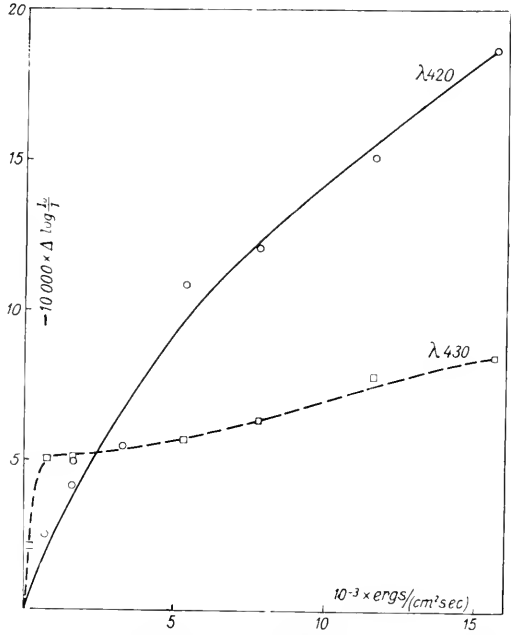

Fig. 4. Changes in *optical density of Rhodospirillum rubrum*, at 420 and 430 mμ as *function of the intensity* of the exciting light in water flushed with hydrogen.

peak is shifted to 790 mμ and the near ultraviolet band to 430 mμ. These shifts may be interpreted as indicating the dehydrogenation of one of the two reduced pyrrole nuclei in bacteriochlorophyll (*cf.* 5).

In anaerobic distilled water the negative maximum occurs at 420 mμ (Fig. 3), suggesting the oxidation of *Rhodospirillum rubrum* cytochrome *c*, a pigment isolated by Vernon and Kamen (3). The hump at about 430 mμ suggests the oxidation of cytochrome 428.

Figure 4 shows that the change at 430 is saturated at a much lower

intensity than the change at 420 mμ. Thus the peak at 420 and the hump at 430 mμ must be caused by different pigments—a conclusion consistent with the suggestion that the changes at 430 and 420 are mainly caused by cytochromes 428 and *c*, respectively. Since thus cytochrome 428 seems to attain saturation at a lower intensity than cytochrome *c*, it may further be concluded that in this experiment oxidation of cytochrome *c* is presumably not caused by oxidized cytochrome 428.

CONCLUSIONS

The experiments reported indicate that cytochrome 428 participates in photosynthesis, since its oxidation-reduction state is shifted considerably towards the oxidized side upon onset of photosynthesis. Also, cytochrome *c* can be oxidized by light, but this oxidation was observed by us only under slightly unphysiological conditions. It is possible, as Chance and Smith (6) concluded, that it occurs to a small extent also in photosynthesizing cells. This conclusion was based on the assumption that the minor band at 550 mμ in the difference spectrum was caused by cytochrome *c*. It may, however, have been caused by a band of cytochrome 428. A curve similar to that of Fig. 4 but measured at 550 mμ would probably establish this point. Vernon and Kamen (3) extracted three cytochromes, one of which was *c*, from *Rhodospirillum* strain 1 and determined the absorption spectra in the oxidized and reduced state. The difference spectrum (oxidized minus reduced) of one of these cytochromes had a maximum at 428 mμ. However, the half-width of the band was 27 mμ, which is different from the 12-mμ half-width of cytochrome 428. The difference spectrum of the third cytochrome was also different from that of cytochrome 428.

The suggestion (4b) that cytochrome 428 was oxidized not only by light, but also by bubbling air through an anaerobic suspension, was confirmed by Chance and Smith (6).

The changes, interpreted above as caused by oxidation of bacteriochlorophyll, were observed only under conditions in which cytochrome 428 was in the oxidized state. It is possible, although not yet proved, that the changes *b* and *d* occurring at high exciting intensities in anaerobic peptone (Fig. 1, graphs III and IV) were also caused by oxidation of bacteriochlorophyll.

The scheme of Fig. 5 is an attempt to explain various observations. A small part of the bacteriochlorophyll is oxidized by light and simultaneously a reduced compound H, which may be a reduced pyridine nucleotide, is formed. Oxidized bacteriochlorophyll oxidizes one or more reduced cytochromes. The oxidized cytochromes cause the oxidation of the substrate and of a small part of H, the last reaction leading to the formation of adenosine triphosphate (ATP), which assists in the reduction of CO_2. The reactions leading to the reduction

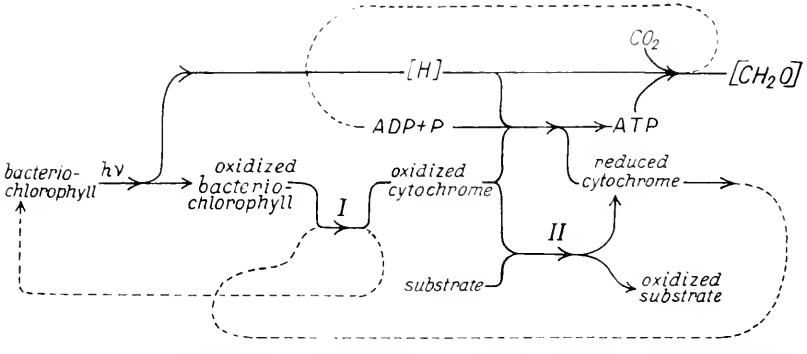

Fig. 5. Hypothetical scheme indicating *role of "active" bacteriochlorophyll and of cytochromes in photosynthesis* of *Rhodospirillum rubrum*.

of CO_2 may be analogous to those in algae. The postulated formation of ATP is in accordance with Frenkel's (7) finding that illuminated extracts of strain 1 produce ATP from ADP and inorganic phosphate. It should be stressed that the scheme is provisional and incomplete.

Acknowledgment. Most experiments reported in this paper were carried out in the Department of Plant Biology, Carnegie Institution of Washington, Stanford, California. I wish to thank Dr. C. S. French for continued interest, advice and valuable assistance, Dr. J. H. C. Smith and other members of the Carnegie Institution for friendly advice, and Mr. J. J. Stekert for technical assistance.

Discussion

Whittingham: Hill has suggested there is a second cytochrome in *Chlorella* and *Porphyridium* which, in the reduced form, has the sharp absorption at 563. You would assume that, if this were reduced in algae and cytochrome were oxidized, you would see some change at 563.

Duysens: The difference spectra of *Porphyridium* measured so far do not show a change in that region. Neither do the difference spectra of *Chlorella*. These experiments do not exclude that cytochrome *b* is participating in photosynthesis, since the changes in the difference spectrum may have been too small to be observable under the present experimental conditions.

Becker: What is the present precision in measuring these differences?

Duysens: The highest precision obtained is 2×10^{-5} unit in optical density, when everything is all right.

With the very dense suspensions the precision is less because, at lower intensities, the signal-to-noise ratio is lower. I think that, with the present apparatus, no greater precision than that mentioned can be obtained. It is not only the noise of the primary phototube current but also other changes which cause variations of the indicating apparatus.

Shibata: We have done experiments just on temperature-produced changes in absorption. With *Chlorella*, we observed some changes like those caused by illumination, for instance, in the 525 mμ band. We have also recorded temperature difference spectra for *Rhodospirillum*. With this bacterium, there appeared to be a slight change between 820 and 880 mμ, which in some ways is comparable to that observed in light-produced difference spectra. I do not wish to say that the difference spectrum produced by illumination is actually a temperature effect, but I cannot exclude that a part may actually be caused by temperature. In our experiments, we maintained one sample at 10°C. and the other at 40°C. For the infrared measurements, we used a sulfide detector. For the work in the visible, we modified a model 11 Cary spectrophotometer. I would like to ask Dr. Duysens whether he noticed any temperature effects similar to ours.

Duysens: In our experiments, there was no general increase in temperature exceeding 5° or 6°C. even after hours of periodic illumination. Our illumination-produced difference spectra changes were quite rapid. They were also reversible within a few seconds.

Shibata: I do not imply that your entire effects are due to temperature changes, but I do not see that you have eliminated temperature effects. Bear in mind that the temperature within the grana during illumination may be considerably above that of the surrounding medium.

Duysens: I can only give indirect evidence that the temperature increase during my experiments had little influence, if any. If there was a pronounced effect, one should certainly have seen an increasing change during illumination, because the temperature would be changing during that time.

Benson: The grana would warm up immediately. They would then transfer the heat to the solution. I do not see that the temperature of the grana should rise continually.

Bassham: One might reach a steady state of temperature where there is a warm area around the grana and the heat is conducted away at a steady rate. It is not inconceivable to me that there would be an initial warming up followed by the attainment of a lower steady-state temperature. Much light energy is being converted into heat within the small volume of the grana.

Duysens: This distribution of the heat will take place, I believe, in 0.01 second or so. If this is true, then you would expect changes in the spectrum to take place

within a time of that order. In general the changes take place in a time of one or several seconds. [Except for Witt's effects.—Ed.]

Frank Allen: I would not expect the temperature effect to be so selective over the spectrum.

Rabinowitch: Dr. Shibata, I wonder whether your effects are not the result of scattering. Your changes might result from so-called anomalous dispersion, which is associated with changes in refractive index in the vicinity of an absorption peak. When you change the temperature, even slightly, you do change the index of refraction of the colored particles, in this case, of the grana.

Frenkel: I feel that the 40° C. temperature maintained by Dr. Shibata in his work is rather unphysiological. I would be quite cautious in interpreting the effects. Though some cultures have been acclimatized to 40° C., normal cultures can only stand about 30° C.

Shibata: I believe the *Rhodospirillum* were unharmed by the temperature.

Duysens: I think we have evidence which shows that the decrease in absorption at 880 mμ in *Rhodospirillum* is not a temperature effect under the conditions of the experiment.

If the change in transmission caused by illumination is measured first with the bacteria in distilled water and then in a culture medium, we get strikingly different results. In the first case there is a very great change in transmission; in the second a slight one. If the change were due to a temperature effect, you would not expect this difference because the temperature effect should be about the same for the two cultures.

Myers: Is that a reversible effect?

Duysens: Completely reversible.

Chance: Did it occur to you that the metabolism would increase at the higher temperature and might therefore give rise to the absorption band that you observe between 400 and 450?

Also, some physical properties of the bacteria might change with the rate of metabolism.

Do you think that you are recording the intensification of an absorption band already present or the shift of a band position with temperature?

Wassink: Is there any harm in assuming that, since these obviously are consequences of the related metabolism, they may be brought about as well by light as by temperature? I mean by a different pathway probably, but just by attacking the same compounds, let's say cytochrome or whatever they are, that are involved in these oxidation-reduction changes.

French: I think everybody would agree that that happens.

Chance: I would like to concur in a suggestion made by Dr. Duysens. We routinely use a gray filter of a density of 4 or 5 between a half-voltage tungsten lamp and cell in order to cause a measurable effect. This light intensity might be of the same order of magnitude as the light which you get from the Cary spectrophotometer, and perhaps sufficient to cause a light effect. If the hotter cells have a sensitivity to light different from that of the colder cells, you might well get just the effects you record: an effect of temperature upon the photochemical reaction and not upon the absorption spectrum *per se*.

Rabinowitch: Let's say, in this light, the chlorophyll molecule absorbs a

quantum of about 2 volts once in 100 seconds, about 0.02 of an electron volt per second. Distributing this energy over 100 molecules gives you a temperature rise of only about a fraction of a degree per second. In a few seconds, you can not raise the temperature of a granum more than a degree, if that.

Strehler: In our circulating system, there was no evidence of a temperature change greater than a few tenths of a degree—for the whole suspension, of course.

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Oxidation of Cytochromes upon Near-Infrared Irradiation of *Chromatium*

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The first observation of the oxidation of cytochromes in photosynthetic bacteria upon illumination was made by Duysens (1) in his studies of absorption spectrum changes in *Rhodospirillum rubrum*. Subsequently, Chance and Smith (2) confirmed and reinterpreted Duysens' observation with a more detailed study of the cytochromes involved. My observations were made on the photosynthetic sulfur bacterium, *Chromatium D*, furnished by Dr. M. D. Kamen.

The bacteria were cultured at 29°C. in an inorganic medium containing sodium sulfide, sodium thiosulfate, and sodium bicarbonate as substrates. Glass-stoppered culture bottles were illuminated by tungsten lamps. In each experiment on the effects of illumination a concentrated suspension of bacteria in growth medium was observed by means of a double-beam spectrophotometer (3). The sample could be irradiated by a near-infrared beam ($\lambda > 700$ mμ) perpendicular to the measuring beams. In other experiments two samples in the dark were compared in a recording spectrophotometer (4). All experiments were performed at room temperature (23° to 27°C.).

Since the suspensions were aerobic after the centrifugation and resuspension necessary for concentrating the bacteria, some suspensions were covered with paraffin oil to obtain anaerobic samples. After 15 to 60 minutes under oil in the dark the absorption of a sample at 422 mμ slowly increased to a new steady-state value. The sample was known to be anaerobic when irradiation caused a large diphasic decrease in absorption (Fig. 1) in contrast to the small monophasic decrease in absorption characteristic of aerobic samples.

The change in absorption spectrum of anaerobic suspensions upon irradiation for about 3 minutes is given by curve *A* in Fig. 2. This difference spectrum has an alpha peak, a beta peak, and a gamma

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peak characteristic of cytochrome difference spectra, reduced minus oxidized. The direction of the change shows that the oxidized form of some cytochrome or combination of cytochromes has a higher concentration when the bacteria are irradiated than when the bacteria are in the dark. In this respect *Chromatium* is similar to *Rhodospirillum rubrum* (1,2). However, in the case of *Chromatium* this difference spectrum does not indicate whether more than one cytochrome is involved.

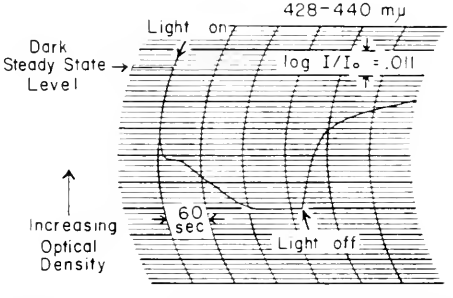

Fig. 1. Typical anaerobic light effect observed at wavelengths between 420 and 430 mμ. In the above record the change in optical density at 428 mμ minus the change at 440 mμ is shown.

The kinetics of this anaerobic light effect answer in part the question of the number of cytochromes involved. The first and second phases of the anaerobic light effect shown in Fig. 1 at 428 mμ were also recorded at several other wavelengths. The spectrum of the first phase is given by curve *B* in Fig. 2; the spectrum of the second phase is given by curve *C*. The positions of the peaks are listed in Table I. The diphasic character of the anaerobic light effect suggests that a chain of intermediates is involved in the photosynthetic transfer of oxidizing equivalents to substrates. Furthermore, the distinct differences between the spectra of the first and second phases indicate that more than one cytochrome is involved. For example, the presence of the 560-mμ shoulder in the spectrum of the second phase demonstrates that a *b*-type cytochrome is involved in the second phase in addition to a *c*-type cytochrome (553-mμ peak) involved in both phases.

Experiments performed with a recording spectrophotometer revealed additional reactions of the *Chromatium* cytochromes in the

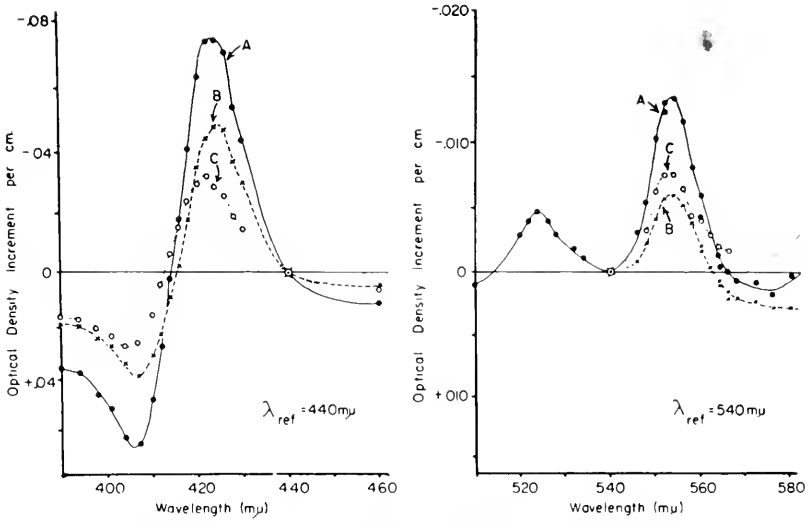

Fig. 2. Changes in optical density of anaerobic *Chromatium* suspensions upon irradiation with near infrared. Curve *A* is the difference between the final irradiated steady state and the dark steady state. Curve *B* is the difference between the intermediate irradiated steady state and the dark steady state. Curve *C* is the difference between the final steady state and the intermediate steady state during irradiation. Curve *C* lies above curve *B* at 553 mμ, but lies below curve *B* at 423 mμ, because a different sample was used for the wavelength interval, 510 to 580 mμ, than was used for the interval, 390 to 460 mμ.

dark. The existence of a CO-binding pigment similar to that found in *Rhodospirillum rubrum* (2) was shown by the addition of carbon monoxide to a suspension in which the cytochromes had been reduced by sodium dithionite. The spectrum of the difference between

TABLE I. Peaks and Troughs of Difference Spectra. Positions of Shoulders Are Shown in Parentheses

Anaerobic light effect			
Total change	423 mμ	524 mμ	553 mμ
First phase	(420)425	?	553
Second phase	422(426)	?	553(560)
Anaerobic minus aerobic	424	524	552
Aerobic light effect	422	?	?
CO minus reduced			
Peak	416		
Troughs	432		551–552

a reduced sample with CO and a reduced sample without CO has a sharp peak and a shallow trough in the Soret region. (See Table I.) This CO-binding pigment may be identical to Vernon and Kamen's (5) *Chromatium* pseudohemoglobin.

Although *Chromatium* is an obligate anaerobe, at least one of its cytochromes becomes oxidized in the presence of oxygen. The spectrum of the difference between an anaerobic sample and an aerobic sample is roughly similar to curve *A* in Fig. 2, but the respective peaks have slightly different maxima as shown in Table I. Also the change in optical density at 422 mμ upon aeration is only about 90% of the total anaerobic light effect. However, irradiation of an aerobic suspension gives an additional decrease in optical density. This aerobic light effect is about 30% of the total anaerobic light effect. These observations indicate that the cytochromes of *Chromatium* are oxidized during irradiation whether the suspension is aerobic or anaerobic. The fact that oxygen completely inhibits the growth of this bacterium cannot be explained simply in terms of its reactions with the cytochromes. The inhibition of growth may be caused by oxygen "poisoning" or by the reaction of the substrates with oxygen outside the bacteria.

The conclusions may be summarized briefly. *Chromatium* contains at least three hemoproteins: a CO-binding pigment, a *c*-type cytochrome, and a *b*-type cytochrome. The *c*-type cytochrome has been partially purified by Vernon and Kamen (5). The anaerobic light effect clearly involves the *c*-type cytochrome and the *b*-type cytochrome. The CO-binding pigment is probably involved also, but its appearance in the anaerobic light effect is masked. In the presence of oxygen the *c*-type cytochrome is oxidized. This oxidation probably requires the mediation of the CO-binding pigment, but the anaerobic minus aerobic difference spectrum does not specifically indicate changes in the CO-binding pigment or in the *b*-type cytochrome upon aeration.

Van Niel's (6) mechanism for photosynthesis in purple bacteria is given by the following equations:

$$4(H_2O + h\nu \longrightarrow H + OH) \tag{1}$$

$$4H + CO_2 \longrightarrow (CH_2O) + H_2O \tag{2}$$

$$2(2\,OH + H_2A \longrightarrow 2H_2O + A) \tag{3}$$

The spectrophotometric studies of *Rhodospirillum rubrum* and *Chromatium* support the concept that a cytochrome system mediates the overall reaction between "OH" and H_2A shown in equation 3.

Discussion

Frenkel: I noticed that the reduction in the dark was relatively slow after the light was turned off. I was wondering if the rate was appreciably different from that in the *Rhodospirillum* which Dr. Chance talked about.

Olson: Yes, it is appreciably different. In my illumination experiments, I often had to wait as long as 10 minutes in order to be sure that the trace had returned to the base line because of that very slow reduction.

Frenkel: How long did Dr. Chance's experiment take?

Chance: A matter of 30 seconds was adequate.

Frenkel: Have you any explanation for that?

Olson: I think that the cytochrome reductase system is perhaps weaker in *Chromatium* than in *Rhodospirillum*.

Linschitz: Was there any fast and slow phase in CO?

Olson: I do not know, because I took only the dark steady-state difference spectrum between reduced samples with and without CO. I observed no kinetics.

Chance: It looks as if, when you turn the light on the CO-binding pigment, this 430-band is oxidized and, in addition, some cytochrome *c*, indicated by the sharp 552-mμ band. The bands that come in slowly are probably due to the remainder of the cytochrome *c* and to cytochrome *b*.

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The Reactions of *Rhodospirillum rubrum* Extract with Cytochrome *c*

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The experiments reported investigated (*a*) the possible role in the respiratory chain of the soluble cytochrome c_2 which has been isolated from the photosynthetic bacterium *Rhodospirillum rubrum* (1) and (*b*) the reactions of extracts of the bacteria on illumination. These experiments represent a continuation of the work of Duysens (2), Vernon and Kamen (3–5), and Chance and Smith (6), which led to the suggestion that one or more cytochromes may be oxidized or reduced in a photochemical reaction. It was hoped that the experiments might shed some light on the oxidation-reduction reactions of the cytochromes, as well as on the possible involvement of these reactions in photosynthesis.

DARK REACTIONS OF EXTRACTS OF *RHODOSPIRILLUM RUBRUM*

An extract of *Rhodospirillum rubrum* was prepared by grinding washed cells with powdered glass. This preparation showed oxygen uptake on addition of a number of substrates. From this extract a particulate fraction was isolated by high-speed centrifugation which took up oxygen only on addition of succinate; the O_2 of the particulate suspension oxidizing succinate approached that of the whole cells. Both the extract and the particles contained the chlorophyll and carotenoids of the bacteria.

When the extract (or particulate suspension) was added to reduced mammalian cytochrome *c* or the bacterial cytochrome c_2 in the dark, a slow oxidation of the cytochrome was observed, as found by Vernon and Kamen (3). The rate of oxidation of mammalian cytochrome *c* was more rapid than that of the bacterial cytochrome c_2. With the

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washed particle suspension, there was no reduction of cytochrome *c* in the dark in the presence of cyanide, which inhibits the dark oxidase, but the cytochrome was rapidly reduced on addition of succinate. Thus the soluble cytochrome seemed to be available for reaction with the enzymes on the particles.

Quantitative measurements were made to determine whether the rate of oxidation of the cytochrome *c* could account for the observed rate of oxygen uptake of the same suspension while oxidizing succinate in the dark, as is observed to be the case with the mammalian respiratory system. Table I shows a comparison of the observed rate of oxygen uptake of the particles oxidizing succinate and the rate of oxygen uptake calculated from the rate of oxidation of cytochrome *c*, assuming that one molecule of O_2 is equivalent to four molecules of cytochrome *c*. The data show that the rate of oxidation of 15 μM cytochrome *c* could account for less than 10% of the respiration; and the rate of oxidation of bacterial cytochrome c_2 is even slower. The data argue against the presence in the bacteria of a cytochrome c_2 dark oxidase system similar to that of mammalian tissues.

TABLE I. Comparison of Observed Rate of Oxygen Uptake of *Rhodospirillum rubrum* Extract with Oxygen Uptake Calculated from the Rate of Oxidation of Reduced Mammalian Cytochrome *c*

Observed oxygen uptake	Oxygen uptake calculated from the rate of oxidation of cytochrome *c*, assuming 1 $O_2 \simeq 4$ cytochrome *c*
47.3 μl./hour	3.4 μl./hour

The same amount of extract was used in the two experiments. The concentration of cytochrome *c* was 15 μM; the mixtures were prepared in phosphate buffer, pH 7.4, 0.1 *M*. In separate experiments, it was ascertained that there was no measurable change in optical density at 550 mμ when the same concentration of *Rhodospirillum rubrum* extract was illuminated in the absence of cytochrome *c*.

LIGHT REACTIONS

The optical density changes at 550 mμ (absorption peak of reduced cytochrome *c*) were followed when an aerobic mixture of reduced cytochrome *c* and *rubrum* extract were illuminated from the side in a special Beckman cuvette designed by Keilin and Hartree (7). The light was filtered to remove light of wavelength shorter than 700 mμ, and the Beckman IP28 phototube was shown not to respond to the filtered light. The optical density changes are plotted in Fig. 1. Illumination produces a rapid decrease in optical density at 550 mμ

(oxidation of cytochrome *c*); then the slow dark oxidation ceases after a period of illumination. This gives additional evidence that the slow dark oxidation does not function in respiration, since oxygen uptake of the extract was found to be unaffected or slightly increased by illumination. As the cytochrome *c* becomes more oxidized following illumination, a slow reduction is observed on cessation of illumination.

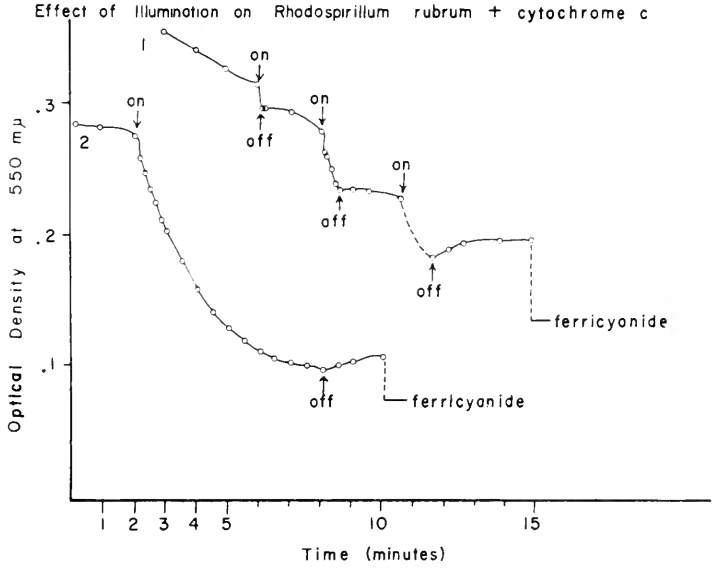

Fig. 1. *Rhodospirillum rubrum* extract was added to reduced mammalian cytochrome *c* at zero time. In curve (1) the cytochrome *c* was 95% reduced and in curve (2) the cytochrome *c* was about 70% reduced at the beginning of the experiment. Illumination was from the side, as described in the text. There was no measurable change in optical density at 550 mμ on illumination of the bacterial extract in the absence of cytochrome *c*. The cytochrome *c* is completely oxidized on addition of ferricyanide.

Also the rate of reduction of cytochrome *c* on addition of succinate in the dark is often increased after a period of illumination. Illumination of the mixture results in the oxidation of the cytochrome *c* until a certain ratio of reduced cytochrome *c* is reached; then no further oxidation takes place. If a mixture of oxidized cytochrome *c* and *rubrum* extract is illuminated, the cytochrome *c* becomes partly reduced. It was found that all the changes in optical density observed

at 550 mμ on illumination of the mixture are not due to the oxidation of cytochrome *c*. Similar results were obtained with mammalian and *rubrum* cytochrome *c* and with either powdered glass or sonic extracts of the bacteria.

The data of Fig. 1 indicate that a soluble cytochrome can be either oxidized or reduced on illumination in the presence of aerobic extracts of *Rhodospirillum rubrum*, the predominant reaction depending upon the state of oxidation of the cytochrome. There is some evidence that the oxidizing and reducing substances can accumulate in solution. For example, there is observed a more rapid rate of reduction of cytochrome *c* in the presence of succinate in the dark after a period of illumination. This view is strengthened by the observation that cytochrome *c* can be oxidized or reduced when the cytochrome in solution is illuminated in the presence of whole cells of the bacteria. It has been shown that cytochrome *c* does not penetrate whole cells.

When the extract of *Rhodospirillum rubrum* was heated at 70°C. for 30 minutes and the mixture suspended in 1.5 *M* sucrose to prevent settling of the precipitate, it was found that cytochrome *c* could still be oxidized on illumination of the mixture. Since this amount of heating will destroy even the most heat-stable enzymes, this means that the cytochrome *c* is oxidized or reduced on illumination in the presence of *rubrum* extract by a nonenzymatic reaction. This eliminates the need for postulating the presence of special "cytochrome *c* photo-oxidase" in the bacteria.

Discussion

Vernon: I should like to ask Dr. Smith if she has taken into account, in this rate constant, the fact that in this organism there is a relatively high cytochrome reductase activity.

Lucile Smith: Not in washed particles. They have no substrate and are completely free of reductase activity.

Vernon: Another point I would like to make is that the effect of heating is similar to what was observed earlier by Kamen and me.

Lucile Smith: Heating to 70°C. for 30 minutes is much more effective than boiling. This will inactivate even horse-radish peroxidase, which is particularly heat-stable. If you heat it this way, you get a fine precipitate, which you can then suspend in strong sucrose solution.

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On the Time Sequence of Reactions in the Anaerobic Light Effect in *Rhodospirillum rubrum**

BRITTON CHANCE, *Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania*

The possibility of participation of the cytochrome c_2 in the photosynthetic activity of *Rhodospirillum rubrum* was suggested by Duysen's spectroscopic recordings (1). We were later able to show with more accurate recordings that several cytochrome or cytochrome-like pigments were affected when the anaerobic bacteria were illuminated with infrared light; we identified absorption bands attributable to cytochromes of types c_2, b, and a "CO-binding pigment" that can act as a terminal oxidase in several bacteria (2,3). It was further shown that oxygen causes spectroscopic effects that are nearly identical to those caused by infrared illumination. This led us to suggest a mechanism for the light effect shown which proposes, among other things, that the oxidizing equivalents produced by the photochemical reaction affect a chain of enzymes, which are very similar or identical to those that are affected by oxygen.

If this mechanism is correct and the oxidizing equivalents react with the chain at the level of the terminal oxidase, then a time sequence along the respiratory chain should be recorded upon illumination of the anaerobic cells. Duysens (1) found the spectroscopic changes to be complete in a time of the order of a second. We already noted that the reaction kinetics, at various wavelengths, were diphasic and that the 430 mμ component responded most rapidly to illumination (2). In the meantime, Olson (4) found that the spectroscopic effects following infrared illumination of *Chromatium* occurred in two distinct phases, and has included in this volume the spectra of the components involved in the rapid and slow effects.

Although the reactions in *R. rubrum* are much more rapid, it has been possible to record at low temperature the spectra corresponding to slow and rapid reactions by means of a sensitive spectrophotom-

* This research is supported in part by the National Science Foundation.

eter that employs two light beams differing slightly in wavelength. These results allow us to determine, on a kinetic basis, the sequence of reactions in the respiratory chain that follows infrared illumination.

METHOD

In our spectrophotometric methods, the "compensating" beam passes over an optical path identical to that of the measuring beam

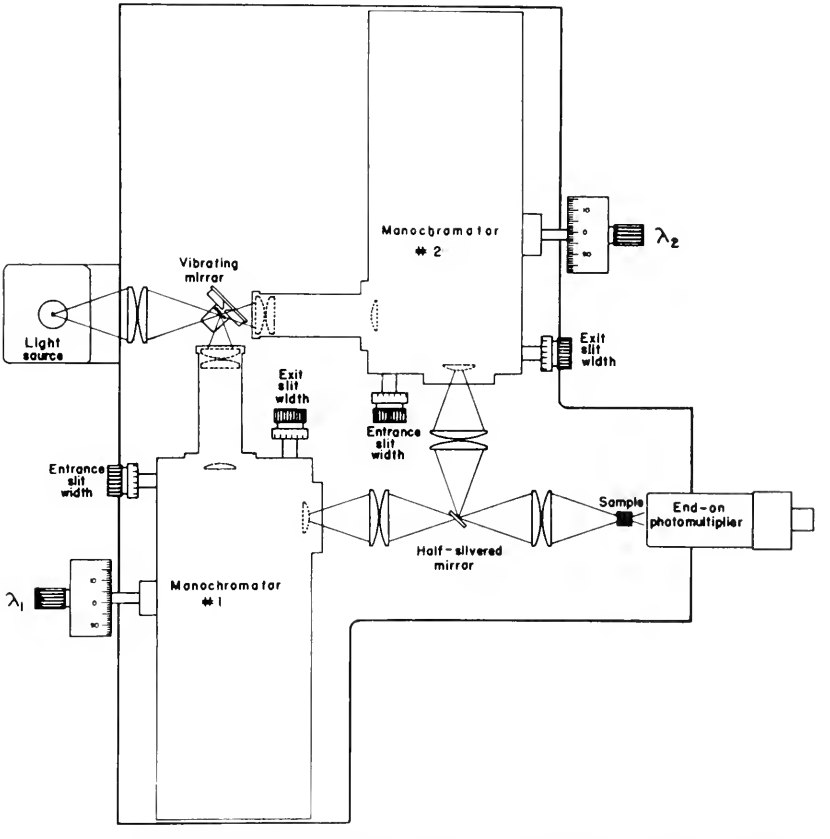

Fig. 1. A double-beam spectrophotometer for recording differences of optical density at two wavelengths selected so as to maximize the response to the oxidation and reduction of the cytochromes and minimize the response to nonspecific effects. This apparatus employs Bausch and Lomb grating monochromators instead of the Beckman quartz monochromators used in previous experiments.

and through the same solution but differs sufficiently in wavelength to give an adequate response to the change in the magnitude of a sharp absorption band (see Fig. 1). We prefer this method to those in which the compensating beam follows a different path from the measuring beam. Concrete evidence of the superior performance of this method is afforded by a comparison of the published records of the effects of illumination upon the cytochromes of *R. rubrum* (1,2). We also show that our signal-to-noise ratio is sufficient to permit a recording of the kinetics of the light reaction and to identify those components that react rapidly. These spectroscopic changes are initiated sufficiently rapidly for the purpose of these studies by a light flash obtained with an electromagnetically operated shutter.

RESULTS

A typical record of the fast and slow phases of the light effect is shown in Fig. 2. The rise of the trace indicates a decrease of light

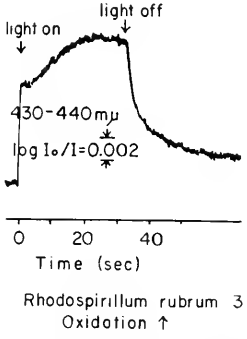

Fig. 2. A typical example of the kinetics of the anaerobic light effect measured in *R. rubrum* at 35°C. The abrupt rise of the trace upon illumination with a low intensity of infrared light, proceeds at a rate that is measurable in recordings at a faster time scale. The optical density changes due to the rapid and slow phases of the effect are plotted as a function of wavelength in Fig. 3. One centimeter optical path.

absorption at 430 mμ with respect to that at 440 mμ. Following the abrupt rise is a nearly stationary state, followed by a much slower rise to a plateau. On turning off the light, the kinetics of the fast and slow effects merge into one another. The slow phase is not completed at the end of the record.

If now one records the magnitudes of the rapid and slow effects caused by illumination of the bacteria as measured at various wavelengths other than the 430 mμ value used in Fig. 2, two difference spectra are obtained, as illustrated by Fig. 3. In the region of the Soret bands, the rapid phase shows a large peak at 428 to 429 mμ with its associated trough at 405 mμ. The spectrum is probably due to the "CO-binding pigment." In the visible region of the spectrum, the peaks are too large compared to the Soret band of the "CO-binding

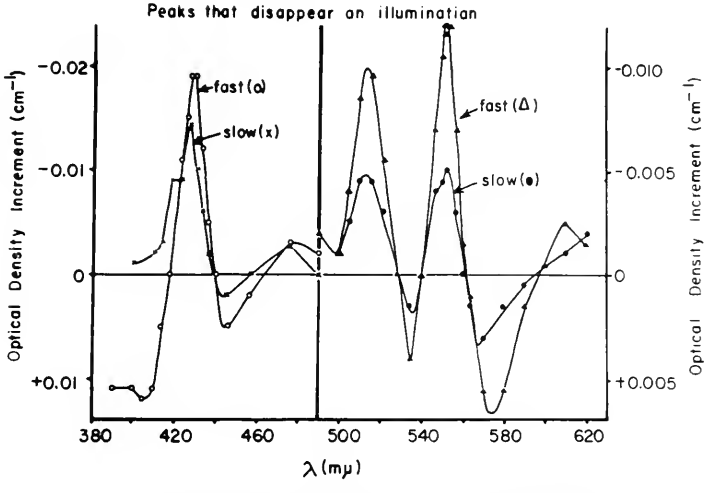

Fig. 3. Difference spectra representing rapid and slow effects following infrared illumination of a suspension of *R. rubrum*.

pigment" to be associated with it, and are, in fact, so large relative to the total change in the Soret region that pigments other than cytochromes may be involved. Nevertheless, there is a superficial resemblance to cytochrome bands, for example, the 550 mμ peak could be attributed to the α band of a "c" type pigment, but the large 514 mμ β band as well as the 574 mμ trough are atypical. The slow phase shows two distinct Soret bands at 420 and 427 mμ attributable to cytochromes of type *b* and *c*, respectively. The Soret bands of these cytochromes are more clearly defined because the deep trough at 405 mμ noted in the rapid phase does not interfere. There is little evidence for the α band of a cytochrome of type *b* in the slower reaction, although this band clearly shows in spectra measured at

room temperature (2) and as a slow component in Olson's studies of *Chromatium* (4).

In summary, the fast light effect involves principally the "CO-binding pigment" and other unidentified pigments, whereas the slow effect involves definitely less of the "CO-binding pigment" and cytochromes of type *b* and *c*. This result confirms the idea that the "CO-binding pigment" is the one most closely related to the photochemical process and, insofar as our data show, is the first hemoprotein to react with the oxidizing equivalents produced in the light reaction. The chain of pigments involved in the anaerobic light effect may be:

(CO-binding pigment) → cytochromes → dehydrogenases

[See Discussion following paper by Britton Chance, Margareta Baltscheffsky, and Lucile Smith, pp. 197–202.]

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The Effect of Hydrosulfite on the Anaerobic Light Effect

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In the course of our studies of the effect of infrared illumination upon the steady-state oxidation-reduction levels of the cytochromes of *R. rubrum*, we have attempted to determine whether the oxidation of cytochromes is prevented by the well-known reducing agent, sodium hydrosulfite (1).

A typical experiment is shown in Fig. 1. The tracings were made in the double-beam spectrophotometer by recording the differences of optical density at 430 as compared with 440 mμ. In the left-hand trace, the aerobic cells exhaust the oxygen dissolved in the solution and become anaerobic. This causes reduction of the hemoprotein absorbing at 430 mμ, as is indicated by the downward sweep of the trace. Illumination with infrared light causes partial oxidation of this pigment and darkness leads to a restoration of the reduced state.

This suspension is then shaken vigorously in order to aerate it and is then replaced in the spectrophotometer. Proof of adequate aeration is afforded by the identical levels of optical density in the initial portions of the two records. Addition of solid sodium hydrosulfite to give a final concentration of roughly 1 mM now causes a rapid reduction that proceeds considerably farther than in the first recording. When the trace has reached an end point, the light is turned on, and again an oxidation of the hemoprotein is obtained. Quantitatively, the optical density change on reduction is 18% greater in the presence of hydrosulfite, while the optical density change on infrared illumination increases 5%. In order to show that an excess of hydrosulfite is present, we made a second addition of hydrosulfite to such a solution and still obtained the oxidation reaction upon illumination. At higher hydrosulfite levels the light effect is diminished. Other evidence in favor of an excess of hydrosulfite is based on the fact that the addition of a low concentration of oxygen by stirring the solution caused no transient oxidation of the cytochromes.

In order to guard against the possibility that the optical density changes caused by illumination of the solution in Fig. 1 (right) involve different pigments from those of Fig. 1 (left), we have repeated the experiment at various wavelengths and have thereby obtained the difference spectrum plotted in Fig. 2. In this spectrum, the optical density changes recorded on turning the light off are plotted. They

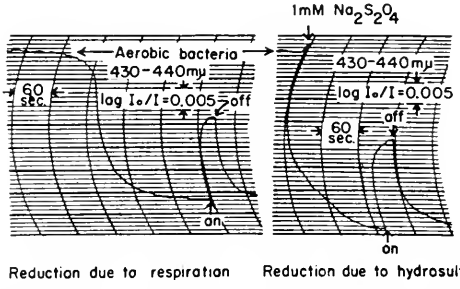

Fig. 1. A comparison of the anaerobic light effect in a suspension of *R. rubrum* in which anaerobiosis has been caused by respiration (left) or by hydrosulfite addition (right). Optical density increases at 430 mμ corresponding to a downward deflection of the trace.

correspond to increases of optical density. This spectrum is essentially the same as that obtained in the absence of hydrosulfite; the same pigments are involved in both cases.

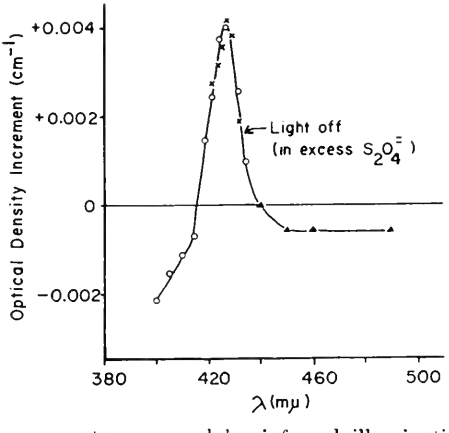

Fig. 2. The difference spectrum caused by infrared illumination of *R. rubrum* suspensions under the conditions of Fig. 1, right.

DISCUSSION

Hydrosulfite readily reduces the ferric forms of hemoglobin, myoglobin, cytochromes, and peroxidases. The only well-documented exception is intact catalase (2). Pigments isolated from *R. rubrum* are reduced (3). Thus we must seek an explanation for the apparent insensitivity of the light effect in *R. rubrum*. There are significant differences in the kinetics of reduction of the hemoprotein in the two records of Fig. 1. On the left, the reduction starts slowly and accelerates to a rapid rate. On the right, the reaction starts at the maximum rate, which is more rapid than in the figure on the left. Thereafter a fairly slow and prolonged reaction ensues which proceeds 18% farther than in the left figure. We attribute the first rapid phase to a combination of a direct reaction of hemoprotein on the cell surface with the reducing agent and to the spontaneous reduction caused by exhaustion of oxygen. The slower phase of the reduction reaction is attributed to the slower penetration of hydrosulfite to more remote parts of the bacterial cell. It is significant that appreciably more hemoprotein is reduced on hydrosulfite addition and that slightly more is available for oxidation by light than in the absence of hydrosulfite.

The result that the light-mediated oxidation of hemoprotein proceeds even in the presence of hydrosulfite gives strong support to the idea that oxygen is not evolved in this photosynthetic process (4,5) and further indicates that transfer of oxygen in a bound form from chlorophyll to hemoprotein is unlikely. A hypothesis in accord with these data is that the oxidant is produced within a structure of chlorophyll and hemoprotein that is inaccessible to the concentration of reductant used here.

[See Discussion following paper by Britton Chance, Margareta Baltscheffsky, and Lucile Smith, pp. 197–202.]

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The Fast Light Reaction of Extracts and of Inhibited Cell Suspensions

BRITTON CHANCE, MARGARETA BALTSCHEFFSKY, and LUCILE SMITH, *Johnson Foundation for Medical Physics, University of Pennsylvania, Philadelphia, Pennsylvania*

As indicated in the preceding paper, the hemoprotein absorption bands, that disappear upon infrared illumination of anaerobic whole-cell suspensions of *R. rubrum*, do so in a two-step reaction that involves different members in the sequence of electron transfer reactions. It was also found that an effect of the opposite sign is recorded when the cells are treated with phenyl mercuric acetate. In this case a strong absorption band in the region of 430 mμ appears when the aerobic suspension is illuminated and this was then interpreted as a photoreduction of hemoprotein (1). On the other hand, Duysens, who has found that aerobic cells treated for some time with distilled water give spectroscopic responses to infrared illumination similar to those caused by phenyl mercuric acetate, proposes that the absorption band that appears upon infrared illumination is caused by an oxidation product of bacteriochlorophyll instead of by a reduced hemoprotein (2).

Extracts of *R. rubrum* prepared according to Frenkel (3) also show spectroscopic effects upon illumination, which resemble those observed in whole cells treated with phenyl mercuric acetate.

This paper compares the various conditions that lead to the appearance of an absorption band in the region of 430 mμ upon infrared illumination and includes some discussion on the nature of the compound observed.

METHOD

The double-beam spectrophotometer used in the studies of the whole-cell suspensions was employed (1). The cross-illumination system used two Wratten 88A filters and a gray filter of density 1 to 5 depending upon the infrared illumination needed to give maximal spectroscopic effect. The monochromatic light intensity was maintained as low as practicable.

Preparation of the materials. The cells were grown as described previously (1). The preparation of the extracts follows as closely as possible the procedures outlined by Frenkel (3). The phosphorylative activity usually corresponded to 22 μM P_i/hour for a preparation that gave an optical density reading of 1.0 at 800 mμ. The reactions were carried out in a 0.2 *M* glycyl-glycine medium of pH 7.4.

RESULTS

Phenyl mercuric acetate treatment of whole-cell suspensions. The first indication of the fast light reaction was obtained by a treatment of the whole cell suspension of *R. rubrum* with 0.1 mM phenyl mercuric acetate (1). The rather complex kinetics of this reaction are

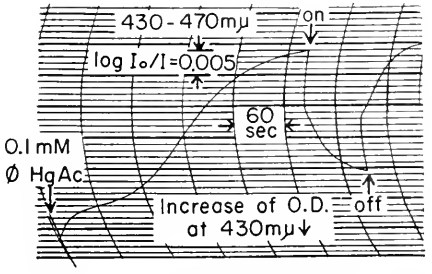

Fig. 1. The time course of the development of phenyl mercuric acetate inhibitor in a suspension of *R. rubrum*. The traces also show the effects of infrared illumination upon the inhibited cells.

indicated by the tracing of Fig. 1. The inhibitor is added to the anaerobic cell suspension and, after a 2-minute lag, the reaction that involves a large decrease of optical density at 430 mμ is complete. This decrease is caused by an oxidation of cytochromes as the dehydrogenase activity is inhibited; and, as previous studies showed, considerably more cytochrome c_2 is oxidized under these conditions (1). Infrared illumination causes an abrupt increase of absorption followed by a much slower reaction and the same sequence of reactions is recorded when the light is turned off. The spectrum corresponding to the fast phase of the light reaction is plotted in Fig. 2 and consists of a sharp peak at 430 mμ* and a trough at 385 mμ.

* One of us (L. Smith) has restudied this effect and obtains a much broader peak at 432 to 436 mμ.

Duysens reports that distilled water treatment of the whole cells gives a similar light effect (2).

After incubation with the inhibitor, the kinetics of the fast light reaction is recorded without interference from the slow reaction as

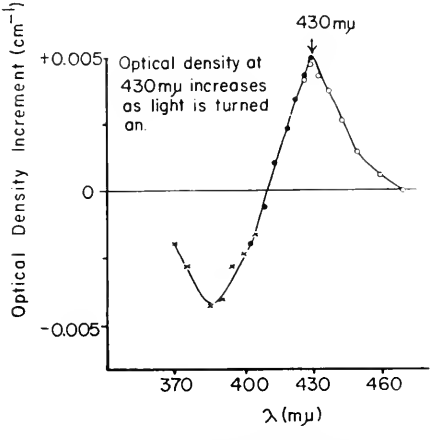

Fig. 2. The difference spectra corresponding to the rapid phase of the reaction caused by infrared illumination in Fig. 1.

shown in Fig. 3. The records show clearly (*1*) that no "instantaneous" processes are involved; (*2*) that the kinetics of the light reaction differ from those of the dark reaction, the light reaction being

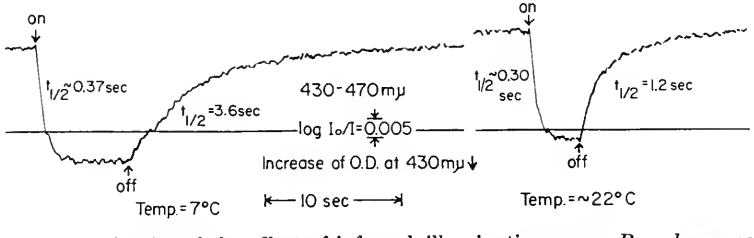

Fig. 3. The kinetics of the effect of infrared illumination upon *R. rubrum* cells inhibited by 0.1 mM phenyl mercuric acetate.

considerably more rapid than the dark reaction, as would be expected if the effect of light is to displace a chemical equilibrium; (*3*) that temperature affects both the light and dark reactions, al-

though the effect upon the latter is considerably greater. Thus the 430 mμ peak is not due to a direct excitation of chlorophyll.

Studies of extracts of R. rubrum. Light effects observed in extracts of *R. rubrum* prepared as described here are shown in Fig. 4. Without any additions to the extract, the light effect is similar to that recorded in Fig. 3 for the whole cells treated with phenyl mercuric acetate. Starting at the left-hand side of the record, a rapid increase in absorption at 430 mμ occurs when the suspension is illumi-

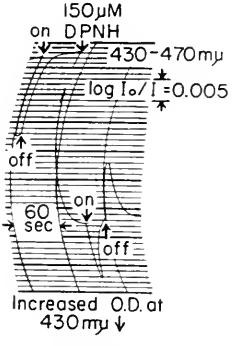

Fig. 4. The effects of infrared illumination upon an extract of *R. rubrum* prepared according to Frenkel (3).

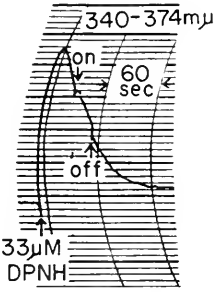

Fig. 5. The effect of infrared illumination upon the kinetics of DPNH utilization by an extract of *R. rubrum*. An increase of optical density at 340 mμ corresponds to an upward deflection.

nated, and a rapid decrease to the original base line is observed in other experiments when the light is turned off. The peak of the difference spectrum for this effect lies at a slightly longer wavelength than that recorded in Fig. 2, but the general nature of the effect is very similar.

If now a reducing agent such as DPNH is added, a large increase of optical density is recorded. The peak of the difference spectrum for the changes caused by DPNH addition is about 428 mμ. This is the expected value corresponding to the reduction by DPNH of the pigment that Kamen has isolated from *R. rubrum* and shown to be reducible by DPNH (4). This pigment is probably identical with the terminal oxidase that we term the "CO-binding pigment" (5). Infrared illumination now causes a further rapid increase of absorption.

but in this case there then follows a slow decrease of optical density which is attributed to oxidation of those pigments reduced by DPNH. On cessation of illumination, the rapid effect is again completed before the slow one gets under way. The oxidation-reduction level then returns very nearly to its initial value.

Effect of light upon DPNH disappearance. In order to determine whether the rate of DPNH disappearance is affected by infrared illumination, we have repeated a portion of the experiment of Fig. 4 in Fig. 5 and have measured light absorption changes at 340 mμ. Addition of DPNH causes an increase of optical density at 340 mμ, recorded as an upward deflection. The utilization of DPNH produces a downward deflection. Illumination causes such a very slight slackening of the rate of DPNH utilization that one may raise the question as to whether DPNH participates in the light-induced reaction. In addition, these effects on DPNH kinetics are irregular; in some cases, an acceleration of DPNH disappearance was noted.

INTERPRETATION

Our current interpretation of the rapid increases of light absorption at 430 mμ following infrared illumination of the cell extracts or of the whole cells treated with phenyl mercuric acetate is as follows: the increased absorption corresponds (*1*) to the reduction of a hemoprotein (*2*) or to the formation of an intermediate compound of chlorophyll, possibly an oxidation product as suggested by Duysens. On the one hand, the peak in the region 430 to 434 mμ suggests hemoprotein reduction (except for the broadness of the band) but, on the other hand, the trough at 385 mμ suggests a disappearance of chlorophyll.

There are a number of arguments against the simple explanation that the effects at 430 to 434 mμ are caused by conversion of all the chlorophyll into a new compound. The magnitude of the spectroscopic change (about 0.025) corresponds to a very small fraction of the total chlorophyll content (<1%, see Discussion), and is not increased with stronger infrared illumination. Such a small extent of reaction is inconsistent with the kinetics of the light reaction which is 4 to 9 times the speed of the dark reaction (see Fig. 3) and should correspond to a large degree of completion of the reaction, i.e., conversion of all the dark form of the 430 mμ pigment. Thus, if chlorophyll is this dark form, only about 1% of the total cell chlorophyll can participate in

this reaction. Interestingly enough, this amount is of the same order of magnitude as the cytochrome content. Some further data obtained, under the conditions of Fig. 3, lead to the possibility that the effect at 430 mμ has somewhat different kinetics from that at 385 mμ; the back reaction at 7° has roughly twice the initial rate of that at 430 mμ. Thus two or more components may be involved, and various hypotheses for this interesting effect are still under active consideration.

Discussion

Granick: In Fig. 3 (p. 187) you had a 520-mμ band that was going up and down. What was that?

Chance: I cannot say it is all due to cytochrome, although a part of it may be attributed to the beta bands of cytochromes *b* and *c;* there are cytochromes which do have relatively large beta bands, for example, cytochrome b_4. Some might be due to the 520-mμ band seen in *Chlorella.* But, of course, the 520-mμ band of *Chlorella* appears upon illumination, whereas that in *R. rubrum* disappears and has no related effects at 475 mμ. It is unlikely that the effect is due to heat for there is the same amount of heat aerobically and anaerobically, whereas there is no spectroscopic effect aerobically and there is a large spectroscopic effect anaerobically. If heat were the cause of these effects, you would expect the same spectroscopic effect aerobically as anaerobically.

Granick: When you add hydrosulfite you reduce the cytochrome at the same time that you remove any oxygen that might be there. Was there something else that showed up by the addition of hydrosulfite?

Chance: Yes, there was a further reduction of cytochrome at 430 mμ. Also this experiment suggests that oxygen is not the oxidant for the light effect.

Frenkel: How long does this hydrosulfite remain in solution as such? Does it decompose very rapidly?

Chance: It is fairly stable for the three-minute interval.

Benson: Does it get into your cells?

Chance: Fig. 1 (p. 190) indicates that it does. It certainly can cause a larger and more rapid reduction of the cytochromes than the endogenous process.

Strehler: Did you say the O_2 is consumed faster than it can be by the endogenous process alone?

Chance: I compared the slopes of the two traces of Fig. 1 (p. 190) and the reduction is more rapid.

Strehler: But there is extracellular oxygen they have to consume.

Gaffron: If you bubble nitrogen through, why do you have to add the hydrosulfite?

Chance: We add hydrosulfite to see if light can cause oxidation of cytochromes in the presence of this reducing agent. The hydrosulfite affects pigments that are presumably inside the cell, and therefore should also be able to react with any oxygen produced in the cell.

Gaffron: What would a little oxygen produced by illumination do? Do you think this would be taken care of completely?

Chance: Yes, by hydrosulfite.

Gaffron: Nobody would really ever expect to get oxygen in *Rhodospirillum rubrum.*

Brown: On the right-hand side of Fig. 1 (p. 190) you are removing oxygen from the environment around the cell. Right?

Chance: Yes.

Brown: Therefore, the cells can effect the internal change much more rapidly than if they have to use up all the oxygen in the environment as they do on the left?

Chance: Somewhat more rapidly.

Brown: You have no evidence, as I see it, that the material penetrates the cell.

Chance: Our evidence is the fact that the spectroscopic shift is greater with hydrosulfite than with anaerobiosis.

Brown: That is significant then?

Chance: The effect is 18% greater with hydrosulfite. With a number of bacteria hydrosulfite addition reveals pigments that are otherwise not reducible under anaerobiosis.

Arnon: To pursue this point, this is a quantitative change on the right, isn't it?

Chance: Yes, this is a quantitative change.

Arnon: Could that mean that you have removed the oxygen more completely here in the presence of hydrosulfite?

Chance: I think we have reduced pigments not reducible under anaerobiosis alone.

Arnon: Will you state the reasons why you don't think the difference could result from the more efficient removal of oxygen on the right and less efficient on the left?

Chance: The respiratory chain is one of the most efficient oxygen removers known; the reaction has not been shown to be reversible.

Gaffron: How far apart are the two records in time? I mean, your base line is not dropping.

Chance: I think this was a couple of minutes. In other words, I shook the suspension to oxygenate it and re-establish the oxidized level, which checks very closely. The base line was not dropping.

Allen: Is there any possibility that the infrared-induced transformation of some of these pigments might be pH-sensitive?

Chance: We have some data from yeast cells obtained by changing the internal pH. If these cytochromes are like the ones in yeast, a spectral shift is unlikely.

Allen: Dr. Hendley has found rather large "gushes" of acid on illuminating some strains of purple bacteria. These very much resemble the changes in transmission that you get initially on turning the light on. I wonder whether there is a connection between the gushes and your absorption changes.

Chance: We would expect a change of pH to affect the pyridine nucleotide because it is in equilibrium with hydrogen ions. In yeast cells we find that the pyridine nucleotides are oxidized when acid penetrates the cells, but the cytochromes are not oxidized or reduced and thus would not respond spectroscopically to the acid gushes. Unfortunately, we don't get any response at 340 mμ with the

Rhodospirillum rubrum, so could not use DPNH as an indicator of intracellular pH changes.

Strehler: Do you get any evidence that a substance other than that from *p*-phenyl mercuric acetate treated cells is being reduced in the light?

Chance: No, not to any appreciable extent in *R. rubrum*. The spectroscopic effects of Fig. 1 (p. 193) observed in the aerobic cells don't appear until an appreciable portion of the cytochrome has been reduced under anaerobiosis.

Frenkel: Have you observed any spectral changes in cell-free preparations?

Chance: Yes, but they are very different from those observed in whole cells. A photooxidation reaction is observed when you reduce the cell-free preparation with DPNH. Also the cell-free preparations show an aerobic light effect similar to that observed in phenyl mercuric acetate treated whole cells.

Frenkel: I just ran an experiment which somewhat reminds me of your reduction in the dark. I refer to the reduction of cytochrome added to a cell-free preparation after the light period. The reaction appears to be almost completely anaerobic, but there must be a small amount of oxygen present because on illumination the cytochrome is oxidized. I don't know whether it goes as you, or as Vernon and Kamen, postulate. The interesting thing is that, after the light is turned off, the cytochrome is reduced at about twice the initial rate. So, this effect resembles the one you obtained.

Chance: Yes, our records show a rapid reduction of the intracellular cytochromes when the light is turned off, and our results with the DPNH-reduced extract are in agreement, too.

Frenkel: I just wondered whether this was evidence for the formation of a reducing power in the light.

Chance: Reducing power is formed, but our data do not say where. Remember that the dark oxidation of DPNH is not slowed by illumination (Fig. 5, p. 195).

Arnon: Did I understand you to say that, in cell-free preparations, you observed reduction rather than oxidation? Is that correct?

Chance: A change at 430 mμ in cell-free preparations is also observed in whole bacteria inhibited by phenyl mercuric acetate. This effect may be due either to a reduction of cytochrome or to an oxidation of bacterial chlorophyll. On the other hand, add a reductant like DPNH to the extract and you will still observe this 430 mμ effect. In this case it will be followed by a slow oxidation reaction. Thus one may question whether a reduction occurs.

Arnon: Will you just define what you mean by 430 effect?

Chance: There are two hypotheses: (1) an oxidation of chlorophyll or (2) a reduction of hemoprotein.

Arnon: Is the reducing effect the last one?

Chance: Yes, although this is a less plausible hypothesis.

Frank Allen: Is it possible to calculate in any way the extent of the change that one would expect in chlorophyll under normal conditions and to compare this with the changes that are observed whose nature is undetermined to see if we are at least in the right ball park?

Rabinowitch: What you mean by "under normal conditions"?

Frank Allen: Any conditions you can do it under. Given an illuminated plant, can we not assume that the changes we observe are related to the number of

molecules that become transformed? Are these changes of sufficient magnitude? I don't know.

Rabinowitch: You want to see the changes in the pigments responsible for the changes of absorption; but if you don't know the absorption coefficient of your pigment you just cannot separate the product into two factors. Therefore, you don't know if you have a very small change of the whole material or if a very small part of the material is transformed into something which has a quite different absorption coefficient.

Lucile Smith: Let us say, if the changes that Dr. Duysens gets and possibly the change observed in the extract of *Rhodospirillum* are changes in chlorophyll, and if you assume that the *Soret peak* of chlorophyll is similar to that of *cytochromes* in that region, there are the changes of the same order of magnitude as the changes in cytochromes.

Duysens: The changes are only 1% of the absorption of bacteriochlorophyll. I think what Dr. Allen wants to ask is whether there is a change of all bacteriochlorophyll molecules or a change in only a fraction of the bacteriochlorophyll molecules which is very large. At present this question cannot be answered with certainty.

Brown: There is a related question. That is, if you attempt to compute potential changes from these spectral differences and you calculate for the total quantity of material present, the change might be very small. If you calculate on the basis of assumed compartmentalization the change can be enormous. I don't think there is any easy way out of that dilemma.

Wassink: Just a comment on this. Under normal light intensities only a small fraction of the chlorophyll will be excited at a certain moment, and you cannot assume that in the presence of an acceptor it stays in the excited stage.

Rabinowitch: Not in the excited, but in the chemically changed state, yes.

Wassink: Can you?

Rabinowitch: Yes, why not? It depends on the lifetime.

Kamen: Dr. Vernon and I isolated the pigment which is presumably identified with the *Rhodospirillum rubrum*. It turned out to have a broad band. The region at 550 mμ, rather than that at 570 mμ, was associated with the 428 mμ peak. It seems to me, therefore, that there should probably not be an anomaly here, at least so far as this is concerned. But it is a broad band and not sharp. It could be that the fast component that you observed, or that the portion of the band at 550 mμ which was fast, could have come from this broad band. You would not see it so easily with the resolution of the apparatus now, but I don't think there is any real anomaly. The hemoproteins in these bacteria are not identical with the classical hemoproteins. Any arguments based on what you would expect from the classical type of oxidase need not be of concern to us now.

Chance: On the other hand, surely there would be a sharp 550 band due to cytochrome *c*.

Kamen: And there is.

Chance: You also state that the 428 might have a broad alpha band.

Kamen: It does have a broad alpha band in the isolated pigment. I don't deny that in the course of the isolation of this pigment we may have changed it considerably because we used rather drastic methods. This can also explain the

potential of about 200 mv., which may not be correct. However, there is a broad band at 550; and, if we had been more careful in isolating the pigment, we could have sharpened the band.

James Smith: The question has been asked about the positions of the bacteriochlorophyll and bacteriopheophytin absorption bands in the middle of their spectra. In ether the positions of the bands, in mμ, and their specific absorption coefficients (given in parentheses), are as follows: bacteriochlorophyll 577(22.9), 530(3.0), 591.5(52.8); bacteriopheophytin 525(31.9), 495(6.5), 384.5(70.6). As we know, the absorption bands may lie at other positions in living organisms.

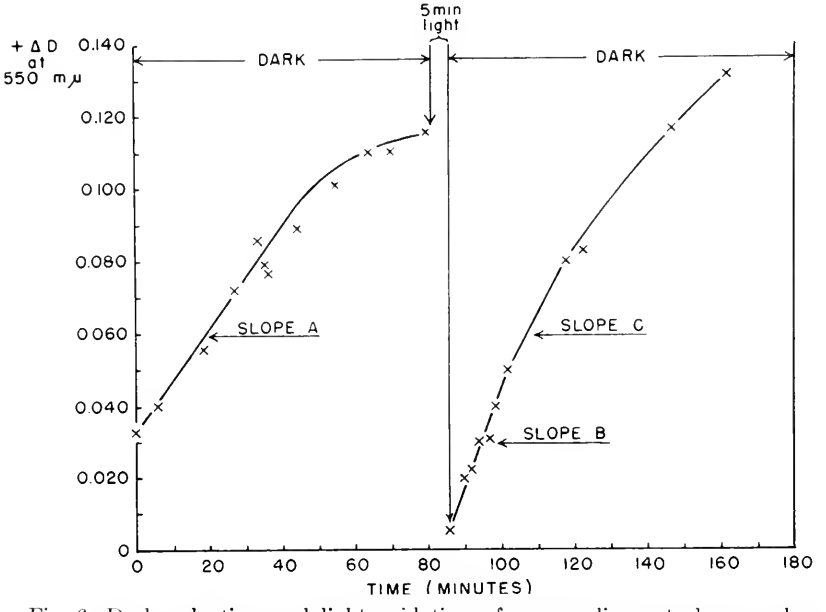

Fig. 6. Dark reduction and light oxidation of mammalian cytochrome *c* by cell-free preparations of *Rhodospirillum rubrum* showing the phenomenon of accelerated dark reduction after a period of illumination.

Frenkel: (*comments added in proof*): I would like to add some observations on the ability of cell-free preparations of *Rhodospirillum rubrum* to reduce added cytochrome *c* in the dark and on the phenomenon of accelerated dark reduction of cytochrome *c* after illumination of this system. Cell-free preparations in 0.2 *M* glycylglycine buffer (pH 7.5) with an optical density of 0.3 at 800 mμ are placed in Thunberg tubes with or without mammalian cytochrome *c* (0.5 μM in a final volume of 3.0 ml.) which are then evacuated. In Fig. 6 above, slope *A* is the initial rate of dark reduction of cytochrome (1.4×10^{-3} optical density unit per minute at 550 mμ). After 80 minutes in the dark, the tubes are illuminated with incandescent light for 5 minutes, which brings about oxidation of the added cyto-

chrome, presumably by a residual trace of molecular oxygen in a manner described by Vernon and Kamen in 1953. Slope B is the initial rate of subsequent dark reduction of the cytochrome after illumination (2.75×10^{-3} optical density unit per minute). Partially purified preparations of the photochemically active particles which are virtually free of cytochrome oxidase activity still show this phenomenon of accelerated dark reduction of cytochrome c after illumination.

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Part IV

"DARK" REACTIONS

1. *Fixation of Carbon Dioxide*
2. *Photoreduction with Various Reductants*
3. *Reduction of Various Oxidants*
4. *Reactions in Chloroplasts and Cell Extracts*
5. *Phosphate Metabolism*

1. *Fixation of Carbon Dioxide*

The "Background" CO_2 Fixation Occurring in Green Cells and Its Possible Relation to the Mechanism of Photosynthesis

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When preilluminated algal cells are brought into contact with $C^{14}O_2$ in the dark, C^{14} is fixed in two different ways (1,2). One is a fixation caused by some photogenetic agent(s), and the other is a background fixation which occurs also in nonpreilluminated cells. It may be reasonable to assume that different agents (or systems of agents) are acting in these two kinds of CO_2 fixation. Let us denote the photogenetic agent(s) by R and the agent(s) acting in the background fixation by D. It has been shown (3,4) that, whereas the reaction between R and $C^{14}O_2$ proceeds very fast and is completed within about 30 seconds at 25°C., the reaction between D and $C^{14}O_2$ is considerably slower. If the radioactivity fixed by D is denoted by r, it is almost a linear function of the time of contact of cells with $C^{14}O_2$ in the dark; namely, $dr/dt=k[D]$, or $r=kt[D]$. The relative concentration of D in nonpreilluminated cells can be gauged by measuring the radioactivity (r) fixed within a definite time of contact of cells with $C^{14}O_2$ in the dark. The level of R in preilluminated cells can be determined by measuring the C^{14} fixation in 30 seconds since, within such a short period, the C^{14} fixed by the background capacity is negligible. If the exposure to $C^{14}O_2$ is longer, say 5 minutes, background fixation becomes significant and the C^{14} fixed will be the sum of a rapid light-induced action of R and a slower almost linear background fixation attributable to D. By subtracting the radioactivity fixed in 30 seconds from that fixed in 5 minutes, we can determine the background $C^{14}O_2$-fixing capacity (on 5 minutes basis) existing in preilluminated cells. Using these methods, we investi-

gated the process of background CO_2 fixation as it is influenced by oxygen and light.

The experimental organism was *Chlorella ellipsoidea* and the apparatus used was virtually the same as that employed by Benson *et al.* (2, see also 4). Algal cells were suspended in 0.02 *M* phosphate buffer of pH 7, and the final concentration of labeled $NaHCO_3$ given to the algae was 6.9 to 9.6 $\times$ $10^{-4}M$. The experimental temperature was 25°C.

EFFECT OF OXYGEN

It was found, in conformity with the earlier findings of Brown, Fager, and Gaffron (5,6), that the level of *D* in the dark was mark-

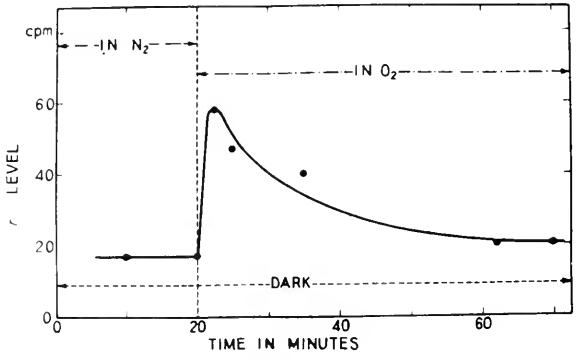

Fig. 1. Dark $C^{14}O_2$-fixation by algal cells that have been kept dark (without provision of CO_2), first in N_2 and later in O_2 atmosphere. Ordinate: cpm of C^{14} fixed in 20 minutes' contact with $C^{14}O_2$ in the dark; abscissa: time of preincubation in the dark with flushing of N_2 and O_2 as indicated.

edly increased when the cells were exposed to oxygen. In the experiment shown by Fig. 1, the algal suspension was first aerated with nitrogen, and at the expiration of 20 minutes the nitrogen was promptly replaced with oxygen, all procedures being carried out in the dark without provision of CO_2. At various intervals during this process, aliquots of algal suspension were brought into contact with $C^{14}O_2$ in the dark, and the radioactivity fixed in 20 minutes was measured. It was found that at the transition from N_2 to O_2 atmosphere, there occurred an abrupt increase in the relative *D* level, which, however, gradually tapered off and eventually attained a

certain steady level. The final r-value established in O_2 atmosphere was always higher than the steady r-level obtained in the N_2 atmosphere. In the particular experiment illustrated by Fig. 1 the level obtained in O_2 was exceptionally low since the value was generally from 2 to 10 times higher than the r level in N_2 (*cf.* Fig. 4). This factor depended upon the prehistory of the algae.

EFFECT OF LIGHT

The effect of light upon the D level was investigated with the results shown in Fig. 2. The cells were first kept in the dark (flushed

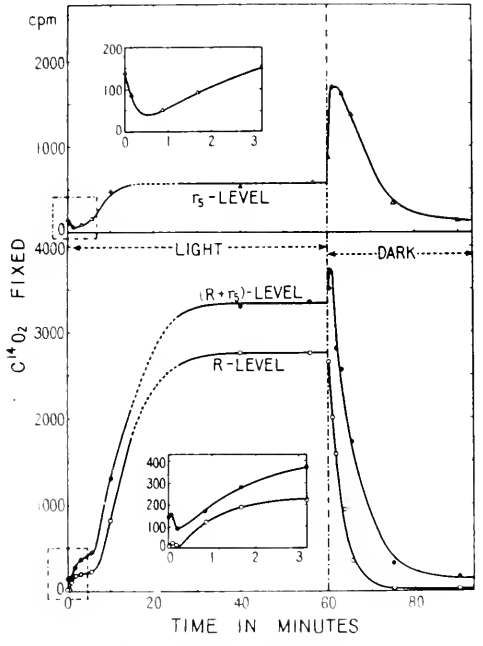

Fig. 2. Effects of transition from dark to light and from light to dark upon the background $C^{14}O_2$-fixing capacity. Measurement of background $C^{14}O_2$-fixing capacity (r_5) was made by subtracting R (i.e., the radioactivity fixed in 30-second contact with $C^{14}O_2$ in the dark) from $R + r_5$ (i.e., the radioactivity fixed in 5 minutes under the same condition). The insets show, on a magnified scale, the events occurring at earlier stages of illumination.

with N_2) for 50 minutes to make them adapt to the darkness and to anaerobic conditions. Immediately after the light was turned on, the

development of C^{14}-fixing capacity was measured, by transferring at intervals aliquots of cell suspension into $C^{14}O_2$ solution kept in the dark, and by measuring the C^{14} fixed within 30 seconds (R), on the one hand, and that fixed within 5 minutes ($R+r_5$), on the other. After 60 minutes of illumination, when the R level was at the stationary state, the light was turned off, and by the same method as above, the course of decay of C^{14}-fixing power was traced. As reported elsewhere (3), the R level showed an induction period with two depressions at the beginning of illumination. When the light was turned off at the stationary state in the light, the decay of R occurred immediately, as it has already been observed by Calvin and Benson (1). A quite different phenomenon was observed in the decay-curve which was measured by 5-minute $C^{14}O_2$ fixation; immediately after cessation of illumination, there occurred an abrupt temporary increase of the C^{14}-fixing capacity, after which the capacity decayed steeply to attain eventually a steady level.

On the upper part of the figure, the difference between the ($R+r_5$) curve and the R curve is plotted. This difference curve, representing the time course of r_5, shows us: (*1*) that on illuminating the dark-adapted algae, the background fixing capacity decreased temporarily, then increased, and eventually attained a steady level, which was considerably higher than the steady level observed in the darkness; and (*2*) that when the light was turned off, the background capacity increased abruptly, and after attaining a certain maximum value, it gradually decreased to a final level which was lower than the steady level observed during the illumination.

PHOTOCHEMICAL CO_2 OUTBURST FROM THE PRODUCT OF BACKGROUND CO_2 FIXATION

An interesting phenomenon was observed when, during the course of dark $C^{14}O_2$ fixation, the cells were subjected to brief illumination. The experimental results are presented in Fig. 3. To the algal suspension which has previously been kept dark for 25 minutes in N_2 atmosphere, $C^{14}O_2$ was added and the course of dark C^{14} fixation was followed. As shown by the curve on the lowest part of the figure, the C^{14} fixation proceeded almost linearly with time when the darkness was continued uninterrupted. When, however, during the course of this process, the algae were exposed to a 1-second light

flash, there occurred a distinct break in the course of C^{14}-fixation, and the subsequent ascension of the curve started from a point which was lower than that observed immediately before the flashing of light. If the duration of flashing light was prolonged to 3 seconds, the break of the curve became less abrupt, but the lowering of the subsequent

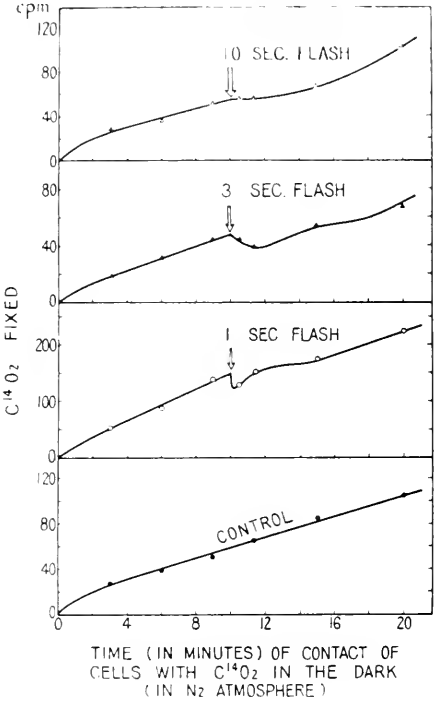

Fig. 3. $C^{14}O_2$ outburst from the product of background $C^{14}O_2$ fixation occurring in N_2 atmosphere. $C^{14}O_2$ was supplied in the dark after the cells were dark-adapted for 25 minutes in N_2 atmosphere. Light flashes of indicated duration were applied at the points shown by arrows.

curve was more pronounced than was the case with the 1-second flash. When the flash duration was 10 seconds, the break of the curve practically disappeared, and the subsequent part of the curve showed a peculiar course, ascending with increasing steepness with the lapse of time. The break in C^{14} fixation time course caused by 1- or 3-second flashes must be taken as evidence that a part of C^{14} fixed

during the preceding period was removed by the effect of brief illumination. Conceivably, there occurred a decarboxylation of some carboxylated compound formed by the preceding background $C^{14}O_2$ fixation. The fact that the breaking effect became more and more

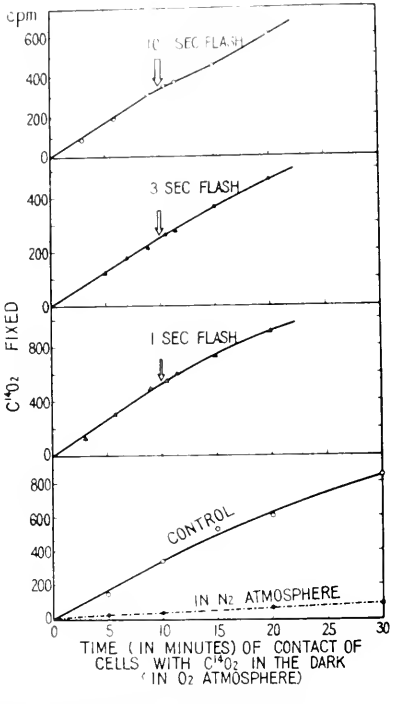

Fig. 4. Effect of light flash upon the background of $C^{14}O_2$ fixation occurring in O_2 atmosphere. $C^{14}O_2$ was supplied in the dark after the cells were dark-adapted for 25 minutes in O_2 atmosphere. Flashing light of indicated duration was applied at the point shown by the arrow.

obscure with the increase of flash duration may be due to the canceling of the effect by the formation of light-induced $C^{14}O_2$-fixing power, which became stronger at longer flash duration.

In the experiment described above, $C^{14}O_2$ was supplied to the algal suspension in the absence of oxygen. When similar experiments were performed in O_2 atmosphere, the results obtained were entirely different. As may be seen from the results shown in Fig. 4, there was no

indication at all for the occurrence of photochemical decarboxylation when algae were given $C^{14}O_2$ in O_2 atmosphere.

DISCUSSION AND SUMMARY

We have seen that the $C^{14}O_2$ fixation by green cells in the dark occurs much faster in O_2 atmosphere than in N_2 atmosphere. By applying flashing light lasting 1 to 3 seconds it was demonstrated that a part of C^{14} fixed in N_2 was photochemically decarboxylated, whereas no such light effect was detectable when $C^{14}O_2$ fixation occurred in O_2 atmosphere. These facts indicate that the processes of dark CO_2 fixation are different under aerobic and anaerobic conditions. Indeed, it was observed by Brown, Fager, and Gaffron (6) that the products of background $C^{14}O_2$ fixation were different according to the presence or absence of oxygen; namely, whereas the C^{14} fixed under anaerobic condition was all found in the water-soluble fraction, those fixed under aerobic condition were both in water-soluble and water-insoluble fractions, being incorporated even in fats, starch, and proteins. In view of these observations our experimental results may be explained as follows. Under anaerobic conditions the products of background CO_2 fixation may remain mostly in the state of rather simple carboxylated compounds, at least part of which are sensitive to photodecarboxylation. In the presence of oxygen, these compounds will be subject to further transformations, the primary step of which may be a reduction or exchange of carboxyl groups, so that the decarboxylating effect of light, if any, will become undetectable by the tracer technique we applied.

By the "subtracting method" we used, it was shown (*1*) that when algal cells were illuminated after prolonged darkness, the background CO_2-fixing capacity decreased temporarily, and then increased gradually to attain a steady level which was considerably higher than the steady level observed in the darkness, and (*2*) that when the light was turned off after continued illumination, the background CO_2-fixing capacity increased temporarily, and, after attaining a certain maximum value, it gradually decreased to a final level which was lower than the steady level observed during the illumination. The temporary decrease of CO_2-fixing capacity occurring immediately after transition from dark to light, as well as the photochemical decarboxylation of some product of background CO_2 fixation, may

probably be related to the CO_2 outburst observed by Emerson and Lewis (7) and to the lag of CO_2 uptake occurring at the beginning of illumination, which was observed by McAlister and Myers (8–10), Aufdemgarten (11, 12), and van der Veen (13, 14). These authors have also observed that when light was turned off after prolonged illumination there occurred a "gulp" of CO_2 or a lag of CO_2 production, which may be accounted for by the temporary increase of background CO_2-fixing capacity occurring immediately after the transition from light to dark. The fact that the background CO_2-fixing capacity is largely influenced by light may be taken as an evidence for its being related directly or indirectly to the mechanism of normal photosynthesis.

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Some New Preillumination Experiments with Carbon-14

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Using C^{14} as a tracer Calvin and Benson (1,2) found that the CO_2-fixing capacity of green cells increased markedly when the cells were illuminated immediately prior to provision of CO_2 in the dark ("preillumination"). This CO_2-fixing capacity gradually decreased in

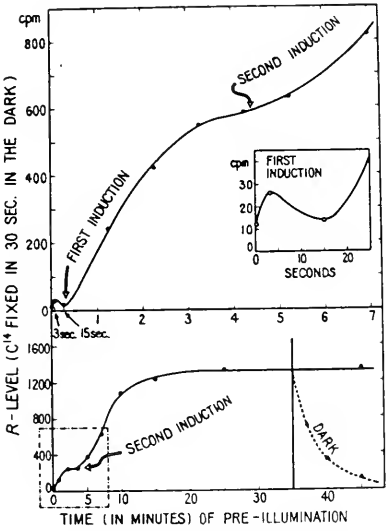

Fig. 1. Induction phenomenon in the process of formation of R in the light. Ordinate: cpm of C^{14} fixed in 30 seconds in the dark; abscissa: the time which the cells spent in the light and dark, as indicated, prior to the provision of $C^{14}O_2$. Experiments illustrated in the upper and lower parts of the figure were performed with different algal samples.

darkness, and it was inferred that during illumination the concentration of the agent in cells responsible for the CO_2 fixation is determined by relative rates of photochemical formation and non-

photochemical decay. We have made studies on the processes of formation and decay of this agent which, for convenience, we shall denote as *R*. In this paper we report the results of experiments which lead to the conclusion that *R* is formed photochemically concomitant with oxygen evolution, reacts with oxygen and quinone, functions as a reducing agent in CO_2 fixation, and is involved in the cyanide inhibition of photosynthesis.

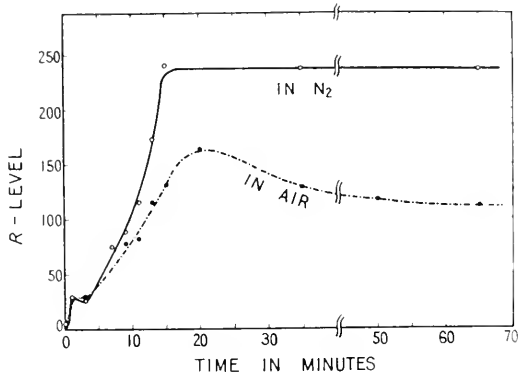

Fig. 2 Effect of air on the course of formation of *R* in the light. Air was introduced 45 minutes before the beginning of illumination.

The experimental organism was *Chlorella ellipsoidea*, which was grown as previously described. In most cases the experimental temperature was 25°C. Preillumination was accomplished while the suspension was flushed with N_2. The $C^{14}O_2$ was provided in the dark in an atmosphere of N_2.

We were able to confirm that nearly all the effect of preillumination is achieved within 30 seconds of light and that background fixation by nonpreilluminated cells is negligible in short time intervals. Consequently the C^{14} fixed in 30 seconds of light is taken as a measure of *R*.

Figure 1 shows that, after prolonged darkness, *R* was formed with a characteristic induction period. Two transient depressions in the time course of *R* increase may be noted—the first occurring at about the 10th to the 15th second, the other between the 2nd and 5th minute. The steady-state *R* level obtained in the light was about 5 to 7 $\times$ 10^{-6} molar CO_2-equivalent per gram dry weight of algae.

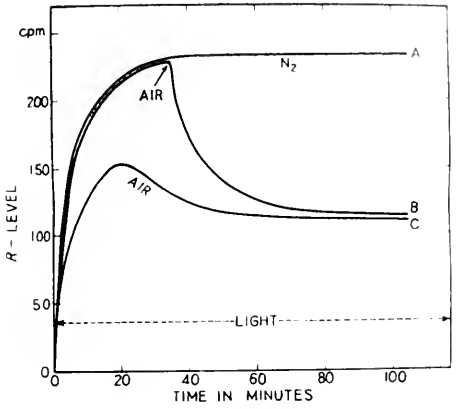

Fig. 3. Effect of air on the course of formation of R in the light. Temperature: 20°C. Curve A: algal suspension was flushed with N_2 throughout the experiment; Curve B: algal suspension was flushed first with N_2 and later (at the time indicated by the arrow) with air; Curve C: algal suspension was flushed with air from the beginning of illumination.

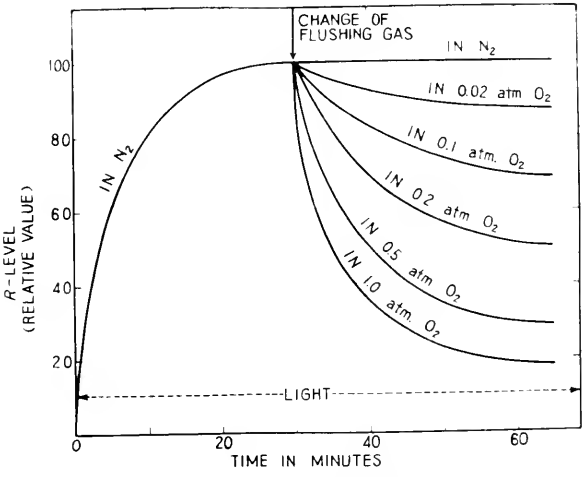

Fig. 4. Effect (in the light) of varying concentrations of O_2 on R pre-formed in N_2 atmosphere.

Decay of R in the dark normally was complete in some 15 minutes. In the presence of oxygen R was considerably depressed. Figure 2 shows that the initial course of R formation (including the char-

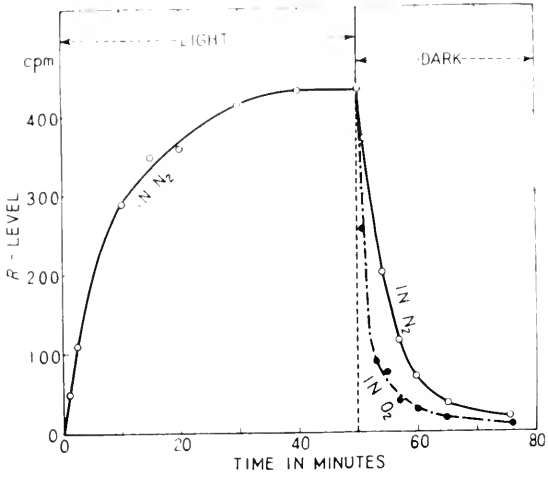

Fig. 5. Effect of O_2 on the decay of R in the dark. Temperature: 20°C. Abscissa: the time which the cells spent in the light and dark, and in N_2 and O_2, as indicated, prior to the provision of $C^{14}O_2$.

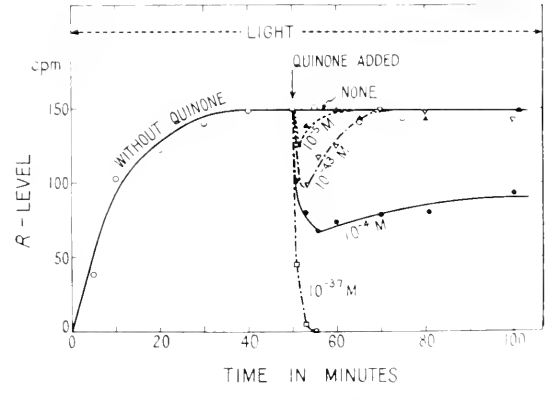

Fig. 6. Effect (in the light) of varying concentrations of quinone on the R-level which had previously attained the stationary value in the absence of quinone.

acteristic second transient) was not affected by oxygen but the final R level was less in air than in N_2. If R was formed anaerobically and the suspension later flushed with air the same final steady-state value of R was obtained as shown by Fig. 3. The effect of oxygen in depress-

ing the R level increased with O_2 partial pressure (Fig. 4) and was reversible.

Figure 5 shows that the decay of R was markedly accelerated by the presence of O_2.

Quinone reacts with R in much the same manner as does O_2 except that, in high quinone concentration, inhibition is irreversible (Fig. 6). The CO_2-fixing power of cells is not restored even by repeated washings with phosphate buffer after light exposure in the presence of

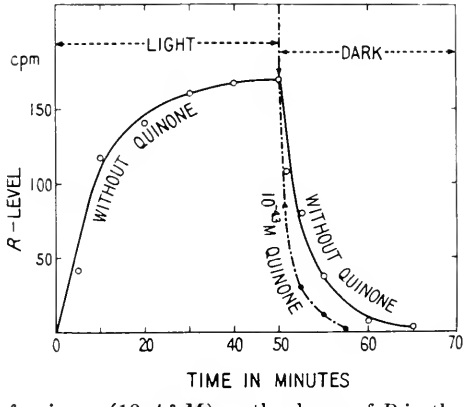

Fig. 7. Effect of quinone ($10^{-4.3}$ M) on the decay of R in the dark. The algae were first illuminated for 50 minutes in the absence of quinone, and simultaneously with turning off the light, quinone was added and the subsequent fate of R was followed by measuring 30-second $C^{14}O_2$-fixation in the dark.

2×10^{-4} M quinone. With 5×10^{-5} M quinone (Fig. 7) the decay of the R level in the dark is greatly accelerated, as is the case for the analogous experiment using O_2 instead of quinone.

When we investigated the effect of cyanide on the processes of formation and decay of R, it appeared that this poison did not affect formation of R, except in so far as the second induction was eliminated. This latter observation corresponds to the observations of McAlister and Myers (3) and of Aufdemgarten (4) on the influence of cyanide on the induction phenomenon occurring in the photosynthetic CO_2 uptake. However, when R was formed by preillumination for 50 minutes in the absence of cyanide and subsequently measured by exposure of the cells to $C^{14}O_2$ in the presence of varying amounts of cyanide, it was observed, in confirmation of

Gaffron *et al.* (5), that the initial rate of dark CO_2 incorporation was cyanide-sensitive. Upon reducing the $C^{14}O_2$ fixation time in the dark to 10 seconds (instead of 30 seconds) the initial rate of CO_2 fixation was determined in the presence of varying amounts of cyanide and at various times after the end of illumination. During the course of

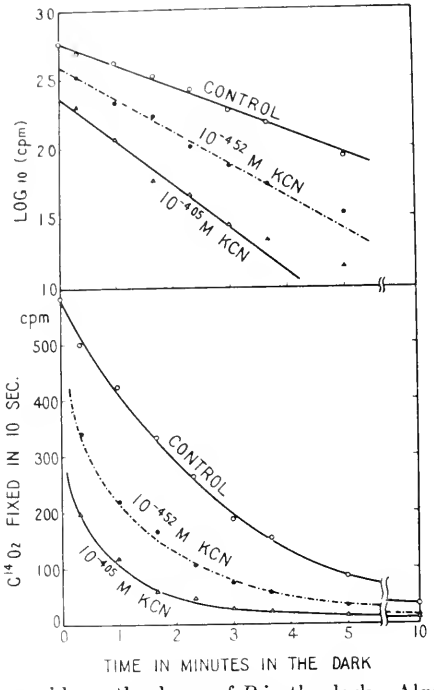

Fig. 8. Effect of cyanide on the decay of R in the dark. Algae were first illuminated for 50 minutes in the absence of cyanide; and simultaneously with turning off the light, cyanide was added and the subsequent fate of R was followed by measuring C^{14} fixed in 10 seconds in the dark.

this 10-second exposure to $C^{14}O_2$ it was determined by other experiments that fixation was linear with time. Figure 8 shows that the decay of CO_2-fixing capacity was an increasing function of cyanide concentration. The upper part of Fig. 8 is a semilogarithmic plot of these data indicating approximately first-order decay.

It can be shown from kinetic considerations that, irrespective of the difference in the rate of reaction between R and $C^{14}O_2$ in the

dark, the tangents of the curves in the above-mentioned semilogarithmic plot represent rates of decay of R in the darkness. The effect of cyanide on the decay of R can be determined by comparing the tangents of these curves. Once the magnitude of the effect of cyanide upon the decay of R is known, the suppressing effect of cyanide on the steady-state R level can be estimated, since this level is determined by the relative rates of formation and decay. The results of this computation are shown in Fig. 9. For the sake of comparison, corresponding data are included showing the effect of cyanide on the

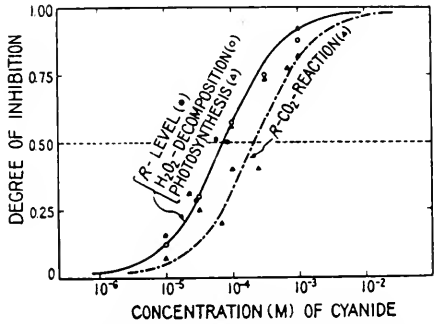

Fig. 9. Concentration-inhibition curves of cyanide acting upon (i) the R-CO_2-reaction, (ii) stationary R-level in the light, (iii) normal photosynthesis, measured manometrically under the condition of light- and CO_2-saturation, (iv) catalytic H_2O_2 decomposition by intact *Chlorella* cells, measured titrimetrically with $KMnO_4$ after bringing the cells into contact with 0.015 M H_2O_2 for 30 seconds. All measurements were made at 25°C. and pH 7.0. The degree of inhibition represents the value $1 - v_g/v$, where v_g and v are the reaction rates or the R-levels in the presence and absence, respectively, of cyanide.

reaction of R with $C^{14}O_2$, on the catalytic decomposition of H_2O_2 by intact *Chlorella* cells, and on normal photosynthesis under conditions of light and CO_2 saturation. From these data we conclude that: (*a*) cyanide has a dual effect upon R; first an acceleration of its decay tending to lower the steady-state level of R in the light, and second, a hindrance of the reaction between R and $C^{14}O_2$, and (*b*) the sensitivity of the R level toward cyanide is greater than that of the R-CO_2 reaction, and indeed it is approximately the same as those of photosynthesis and of the catalytic decomposition of H_2O_2.

We have seen that there are three kinds of reagents whose presence entails more or less marked decrease of the concentration of R in the

algae, viz., (*1*) CO_2, (*2*) oxidants such as quinone or oxygen, and (*3*) cyanide. It is unlikely that these agents of entirely different nature would act upon *R* by the same chemical mechanism. The paradoxical facts can, however, receive a coherent explanation if we make the following assumptions:

a. *R* is a reducing agent which is formed photochemically from its precursor *P* (an oxidized form of *R*) by a reaction accompanied by a liberation of oxygen, such as:

$$P + H_2O \xrightarrow{\text{light}} R \text{ (or } PH_2) + {}^1/_2O_2 \qquad \text{(I)}$$

b. *R* reacts with oxygen in the manner:

$$R + O_2 \rightarrow P + H_2O_2 \qquad \text{(II)}$$

followed by the catalytic decomposition of H_2O_2:

$$H_2O_2 \xrightarrow{\text{catalase}} H_2O + {}^1/_2O_2 \qquad \text{(III)}$$

and, in so far as catalase functions normally, the latter process occurs much faster than the former, so that the overall reaction proceeds according to:

$$R + {}^1/_2O_2 \rightarrow P + H_2O \qquad \text{(IV)}$$

c. *R* reacts also with other oxidizing agents such as quinone (*Q*) and H_2O_2 in the manner:

$$R + Q \rightarrow P + QH_2 \qquad \text{(V)}$$

$$R + H_2O_2 \rightarrow P + 2H_2O \qquad \text{(VI)}$$

d. In the presence of CO_2, *R* is involved in the following sequence of reactions which were postulated by Bassham, Benson *et al.* (2):*

$$CO_2 + \text{RDP} \rightarrow 2\text{PGA} \qquad \text{(VII)}$$

$$2\text{PGA} + 2R \rightarrow 2\text{TP} + 2P \qquad \text{(VIII)}$$

$$2\text{TP} \longrightarrow {}^6/_5\text{RDP} \qquad \text{(IX)}$$

$${}^1/_5\text{RDP} + {}^1/_5CO_2 \rightarrow {}^2/_5\text{PGA} \qquad \text{(VII)}$$

and among these reactions (VIII) is the rate-determining step,† so that the overall reaction proceeds according to:

$$R + {}^3/_5CO_2 \rightarrow P + {}^1/_5\text{PGA} \qquad \text{(X)}$$

* Abbreviations used: RDP for ribulose-diphosphate, PGA for phosphoglyceric acid, and TP for triose-phosphate.

† Relative rates of these reactions are assumed to be in the order: (VII) > (IX) > (VIII).

e. Under ordinary experimental conditions (i.e., in the concentration range occurring in ordinary experiments), the reactivity of reagents acting upon R increases progressively in the order: $O_2 < CO_2 < H_2O_2$, quinone.

f. As corollaries of these assumptions we postulate: that the "spontaneous" decay of R (occurring in N_2 atmosphere) is a result of reaction (IV) caused by the endogenous oxygen formed in reaction (I),* and

g. That the Hill reactions occurring in the presence of *exogenous* O_2 or quinone are results of reactions (I) and (II) and (III) or (I) and (V).†

On the basis of these assumptions we can explain the following assemblage of experimental facts:

1. The decay (in the dark) of R occurs even in N_2 atmosphere, but is accelerated markedly in the presence of exogenous oxygen or quinone.

2. Cyanide accelerates the decay of R, because it inhibits reaction (III) by inactivating catalase, so that R is consumed by reactions (II) and (VI) (instead of by reaction (IV) as is the case in the absence of cyanide).

3. When R reacts with $C^{14}O_2$, the C^{14} fixed is almost entirely incorporated in the carboxyl group of phosphoglyceric acid (and partly in that of pyruvic acid), as was observed by Gaffron *et al.* (5).

4. The rate of photosynthesis, which is initiated by reaction (I) followed by reaction (X), is decreased in the presence of cyanide and oxygen (7,8), and the depressing effect of these substances is stronger when the concentration of CO_2 applied is low (because of the competition between CO_2, O_2, and H_2O_2 for R).

5. The Hill reaction caused by quinone is not inhibited by cyanide

* In reaction (I) oxygen is supposed to be formed in direct proximity to R, so that it reacts readily with R without being removed by such procedures as bubbling of algal suspension with helium or N_2.

† It may be argued that this statement is contradictory to the observation of Mehler (6), who, using isolated chloroplasts, could not demonstrate the aftereffect of preillumination to provoke the reduction of Hill reagent (2,6-dichlorophenol-indophenol). It should, however, be pointed out that in his experiment the preillumination lasted only $^1/_2$ to 2 minutes, which, according to our observation, is such a short time as to produce only $^1/_{20}$ to $^1/_6$ of the stationary R level to be established in the light. Conceivably, the insufficiency of the length of preillumination was the cause of the negative result obtained in Mehler's experiment.

and the oxygen does not compete with quinone in their action as the Hill reagent (6) (because R reacts with quinone preferably to oxygen, and, therefore, there is little chance for the formation of H_2O_2).

6. In normal photosynthesis (occurring under the condition of light and CO_2 saturation), existence of O_2 does not lead to the formation of H_2O_2 detectable by the trapping method using catalase and ethanol (because, under the said condition, not only is catalase actively functioning, but also R has a stronger reactivity toward CO_2 than toward O_2).

7. When oxygen acts as a Hill reagent, intermediate formation of H_2O_2 is detected by the trapping method, and on trapping of H_2O_2 there occurs a positive O_2 uptake, which does not occur—irrespective of the presence or absence of cyanide—when H_2O_2 is not trapped (*cf.* reactions (I), (II), (III), and (VI)). Along the line of our reasoning mentioned under (*e*), it is deduced that both the stationary level of R in the light and the process of its decay in the dark will vary in a wide range according as the kind of reactant existing in the medium. The state of affairs may give us a clue to the understanding of the following facts:

8. Whereas the induction period in the formation of R (in the absence of CO_2 and exogenous O_2) lasts as long as 20 to 30 minutes (see Fig. 1), the induction period occurring in normal photosynthesis (in the presence of sufficient quantity of CO_2) is of much shorter duration (2 or 3 minutes).

9. In the absence of CO_2 and of exogenous O_2, the decay of R in the dark occurs with a half-life of about 2 minutes, whereas the dark C^{14} incorporation after a period of steady-state photosynthesis in the presence of CO_2 decays much faster (with a half-life of only 3 seconds; Gaffron *et al.* (5)).

10. In the Hill reaction occurring in the presence of quinone, practically no induction period is observable.

The role of catalase in the mechanism of photosynthesis has long been a point of dispute among investigators of photosynthesis. As we have shown, cyanide not only inhibits the reaction between R and CO_2 but also accelerates the decay of R. The latter effect we attribute to the reaction of R with H_2O_2 which we assume to derive from the reaction between R and O_2 (endogenous or exogenous) and is left undecomposed if catalase is inhibited by cyanide. Since the re-

action between R and O_2, through which H_2O_2 is formed, will be suppressed in the presence of high concentration of CO_2, the existence of catalase would have only a minor significance for the process of photosynthesis occurring under the condition of high CO_2 concentration and low O_2 tension. Even under such circumstances, however, cyanide will inhibit photosynthesis, because it retards, besides inhibiting catalase, the reaction between R and CO_2 and shifts the competition between CO_2 and O_2 in favor of the latter, so that there will occur the formation and accumulation of H_2O_2 which acts upon R leading to a decrease of the rate of photosynthesis. In the photosynthesis occurring under natural conditions, the concentration of CO_2 available is usually suboptimal and moreover, there is a large amount of O_2 in the circumambient medium. Under such a condition, the competition between CO_2 and O_2 for R will be more or less in favor of O_2, and, therefore, there will be ample possibility for the formation of H_2O_2. According to our notion it is to protect the photosynthetic machinery from such contingent accumulation of H_2O_2 that catalase is playing a role in the mechanism of photosynthesis.

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Photosynthesis by the Etiolated Plant after Exposure to Light*

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Except for chlorophyll synthesis, measurements of the acquisition of photosynthetic ability by the etiolated plant after exposure to light have not been extensive. Oxygen evolution by etiolated barley plants commences about 30 minutes after plants are placed in the light (2,3). However, Irving (4) reported that greening etiolated barley and bean seedlings did not fix CO_2 until after they had developed a large part of their chlorophyll.

Observations have now been made on the fixation of $C^{14}O_2$ and the products formed during increasing time intervals after an etiolated Thatcher wheat plant has been placed in white light. Except for the initial photo-conversion of protochlorophyll to chlorophyll, chlorophyll synthesis did not begin until the plants had been in the light for about 2 hours. The rate of $C^{14}O_2$ fixation was almost zero for about 2 hours after rapid chlorophyll synthesis had started, i.e., after 4 to 5 hours of continuous light.

The products formed from the newly fixed $C^{14}O_2$ were analyzed by paper chromatography. During the first 4 hours after the etiolated plants were placed in the light, the only $C^{14}O_2$ fixation was CO_2 exchanged into the carboxy groups of malic, aspartic, and glutamic acids. After 4 hours of light, there was no significant photosynthetic CO_2 fixation even though the plants contained appreciable amounts of chlorophyll. Between 4 and 5 hours of light a preponderant amount of the $C^{14}O_2$ was found in phosphoglyceric acid and alanine. Plants at this stage of greening were apparently not able to reduce the C_3 compounds, first produced photosynthetically, to sugars. This condition in the plant existed for an interval of 1 to 2 hours around the

* Work performed under U.S. Atomic Energy Commission Contract No. W-7405-eng-26. A more detailed description of this work has been published elsewhere (1).

fifth hour of light. After exposures of the plant to light for longer than 5 hours, quantities of C^{14} began to appear in the sugar phosphates and sucrose. The percentage of C^{14} going into sucrose increased gradually during further greening until it had reached a constant value after the etiolated plants had been illuminated for 9 to 12 hours. However, after 12 hours of light, the rate of $C^{14}O_2$ fixation was still only 15% to 20% that of a fully greened plant after 32 hours of light exposure. Thus, by the end of 12 hours, the reactions in the reduction of phosphoglyceric acid were functioning at about equilibrium conditions of normal steady-state photosynthesis. The increase in total fixation rate after 12 hours might be the result of other, more slowly developing processes, such as formation of more chlorophyll and of the CO_2 acceptor, ribulose diphosphate.

Only traces of C^{14} activity were present in the ribulose diphosphate or sedoheptulose phosphate from the fifth to the twenty-fifth hour analyses after illumination of the plant. Thus one reason for the lag in CO_2 fixation rate during the greening period and for the low efficiency of the newly formed chlorophyll was the failure of the plant to adapt to synthesis of the ribulose diphosphate needed to drive the photosynthetic carbon cycle. When etiolated plants were sprayed with ribose prior to illumination, the amount of $C^{14}O_2$ incorporation into the various compounds was increased, but the time sequence when the greening plant could incorporate $C^{14}O_2$ into these compounds was not greatly changed.

One of the last pathways to be activated during the greening process was that for synthesis of serine. About 20 hours of light was required before large amounts of $C^{14}O_2$ began to be incorporated into serine. This was at a markedly later time than that for labeling alanine. It has already been shown that glycolic acid oxidase activity increased greatly in etiolated plants after about 20 hours of light (5) and that glycolic acid is readily converted to serine in plants (6,7). Thus a pathway from the sugars of the photosynthetic carbon cycle, through glycolic acid to serine, appears to be a major pathway for serine synthesis, which occurs late in the greening process.

The very short lag phase in CO_2 fixation (seconds to a few minutes) by a green plant, when transferred from dark to light, indicates the rate at which steady-state photosynthesis is reached in a system already containing chlorophyll and all the enzymatic components for photosynthesis. That can be compared to the >24-hour lag in photo-

synthesis during the greening of an etiolated plant and the stepwise acquisition of the ability to synthesize the photosynthetic organic products. The induction period in photosynthesis by a fully grown plant may be a measure of the rapidity with which a plant can build up the pool size of carbon compounds provided all the enzymes are present. The slow stepwise build-up of photosynthesis during greening of an etiolated plant would appear to be limited by other processes such as synthesis of adequate amounts of enzymes to catalyze some of these reactions.

Discussion

Rosenberg: Is there any evidence from your work, or from previous work in the literature, that in the early stages of development of the photosynthesis apparatus the ratio of CO_2 to oxygen is higher than the steady-state value, as might be the case if reduction to triose did not occur?

Tolbert: No. Rather a literature comparison indicates that oxygen evolution begins before CO_2 fixation, which would make this ratio smaller than the steady-state value.

Gaffron: Did you say that oxygen development starts earlier than CO_2 fixation?

Tolbert: Unfortunately, all the investigators concerned have used different plants. Dr. Thomas, in Utrecht, has measured oxygen evolution during the greening process, and both he and Dr. Smith get oxygen evolution fairly soon after chlorophyll formation begins.

James Smith: It took pretty nearly an hour to get very much though.

Tolbert: This would still be sooner than CO_2 fixation, which did not begin for 4 hours.

Gaffron: It is very interesting that the plant learns to do full photosynthesis by steps.

Fuller: I think that is a very important point. There are other ways that you can control enzyme formation in photosynthesis. For instance, you can divorce enzyme formation from the greening process of the plant. We have grown *Chlorella variegata* in the light on organic substrate, and it does not produce any active carboxylation enzyme. If the endogenous organic substrate is removed then the enzyme is rapidly formed. We have also found that by growing *Euglena* in the presence of acetate in the light, where no chlorophyll is lost, carboxylation activity is strongly suppressed. There are other ways of inactivating photosynthetic enzymes, so it is not the greening process itself but it is the pathways of metabolism, as Dr. Tolbert points out, that control enzyme formation.

James Smith: I might say that steps in the oxygen evolution can be built up in the dark. If you illuminate for 5 minutes and transform the protochlorophyll to chlorophyll, then put the leaf back in the dark for 2 hours, you get only a very slight amount of oxygen evolution. But if you illuminate the leaf again in the air and then measure the oxygen it just streams off. So there is a second photochemical activation in this. In continuous light, this goes on all the time. But there is some-

thing that goes on in the dark that is not photochemical which then can be transformed by the photochemical trigger.

Tolbert: An additional point of interest is that the etiolated plant and the greening process are not sensitive to ionizing radiation. You cannot prevent greening and normal development of the photosynthesis apparatus by massive dosages of gamma radiation of the order of magnitude of 100,000 to 300,000 r.

Lumry: Is there any evidence of spectral chlorophyll changes? As you convert protochlorophyll to chlorophyll can you increase the density of the chlorophyll?

James Smith: Well, you do get this change that I showed here, and whether it is a change in the chlorophyll or whether it is a change in the environment that causes the change in the spectrum, I don't know.

Lumry: Is it ruled out that the spectrum shifts that we have observed in chlorophyll *in vivo* are due to organization of chlorophyll molecules? Must this now be assumed to be a function of the absorptive act for each chlorophyll molecule or can it be due to the nearby neighboring chlorophyll molecules?

I was very much worried by Rabinowitch's and Jacob's conclusion (which I think was their conclusion anyway) in the *Journal of Chemical Physics* that this is not the kind of change—that we would observe too big a change in the spectrum, too much of a shift toward the red, if this was due to dipole-dipole interaction between neighboring chlorophylls and the like.

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Excretion of Glycolic Acid by *Chlorella* during Photosynthesis*

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Chlorella cells that had fixed $NaHC^{14}O_3$ for a few minutes were found to excrete 3% to 10% of the fixed C^{14} as glycolate-C^{14} into the aqueous nutrient medium. Glycolate was the only organic anion excreted which could be detected and analyzed by paper and column chromatography. Analysis of the soluble cellular constituents by paper chromatography revealed the expected photosynthetic products inside the cell. During steady-state photosynthesis, 10 to 100 times as much glycolate was in the supernatant as inside the cells. Normally, 3 to 8 mg. of glycolate per liter of cultures was obtained from actively growing algae. Lewin† has recently found about twice as much glycolate in the media from cultures of *Chlamydomonas*.

The rapid excretion of glycolate by *Chlorella* is dependent on all the factors influencing active bicarbonate fixation: (*a*) the presence of bicarbonate, (*b*) aerobic conditions, and (*c*) light for active photosynthesis, since there must be a net bicarbonate uptake. In addition, the younger cultures in the more active phases of growth and photosynthesis excrete a proportionately larger amount of glycolate. In media above pH 5.5, the excretion is at a constant percentage rate of the total fixed, whereas in more acid cultures the excretion of glycolate decreases until at pH 2.5 it becomes zero.

Glycolate excretion and absorption may represent a glycolate-bicarbonate anionic exchange across the *Chlorella* cell wall without the necessity of a similar cationic shift. Since bicarbonate uptake during photosynthesis and excretion during respiration represent a major ionic movement, a balance of it with some other diffusible anion would lessen an undesirable loss or absorption of cations. If a Donnan

* Work performed under U.S. Atomic Energy Commission contract No. W-7405-eng-26. A detailed report on the identification of glycolic acid and environmental conditions affecting this excretion is being published elsewhere.

† Lewin, R. A., personal communication.

equilibrium exists across the cell membrane, a change in bicarbonate ion concentration should result in an opposite movement of glycolate ion to help restore the following equality:

$$\frac{(\text{glycolate}^-)_{\text{cell}}}{(\text{glycolate}^-)_{\text{medium}}} = \frac{(HCO_3^-)_{\text{cell}}}{(HCO_3^-)_{\text{medium}}}$$

The enormous rate of glycolate-C^{14} excretion during active bicarbonate uptake of photosynthesis indicates that this organic anion is uniquely able to respond quickly to the upset of equilibrium when bicarbonate ion in the cell suddenly diminishes in concentration and more of this anion moves in from the medium.

That glycolate should be the anion functioning in this manner may be the result of several factors. It is a small organic acid, and yet it is one of the strongest acids associated with the cell. It is somewhat unique in being readily available from the photosynthetic carbon cycle. In this respect, it is believed to be formed from a side oxidation pathway from this cycle, starting from a C_2 complex at the oxidation level of glycolaldehyde.

The excreted glycolate does not accumulate in the *Chlorella* culture medium in large amounts, but rather it is rapidly reabsorbed by the algae when one or more of the conditions for its excretion are not met. This accumulation inside the cell, in relatively large amounts, is about equal to the total normally found in the medium, but it should not persist for a long time, since glycolate is metabolized to glycine and serine (1). This factor could contribute to induction effects in photosynthesis after periods of darkness of about 1 hour or longer. Little glycolate would then be left in the cell to exchange rapidly for bicarbonate ion. When the algae are placed in the light, a photosynthetic induction period would ensue while the glycolate pool was replenished by a slow rate of photosynthesis or from reduction of glyoxylate arising from glycine (2) or from isocitrate (3,4).

Other consequences of glycolate excretion during photosynthesis, and its reabsorption and accumulation inside *Chlorella* during dark respiration, have not yet been evaluated. This bicarbonate-glycolate shift may be involved in acid bursts, pH shifts, CO_2 bursts, and quantum efficiency as calculated from short-time or flashing-light experiments.

Discussion

Gibbs: I want to comment on the *glycolic acid excretion*, since I remember that at the botanical meetings last summer Dr. Ranson had some very interesting data on this point. He showed that with normal CO_2 concentrations and *Bryophyllum* one obtains a typical radiochromatogram as shown by many workers. However, as one increases CO_2 concentration up to 30% and 40% the compounds which one normally finds labeled, that is, the free sugars, sugar phosphates, etc., disappear and the only compound which contains tracer is malic acid. As a consequence one might say the carboxylating enzyme is apparently more sensitive to CO_2 concentration than the malic enzyme. One wonders if the ribulose diphosphate that accumulates is partially converted to a diose which cannot carboxylate since the carboxylation enzyme is inhibited so that it spills out of the cell as glycolic acid.

Tolbert: Ranson's work was at 30% to 40% CO_2 partial pressures and he did not report glycolic formation under those conditions. The bicarbonate-glycolate shift occurs at low CO_2 partial pressure, in the range of 1% CO_2 to 0.3% or lower. Even at 2, 3, or 4% CO_2 in air, variations in the path of the carbon have not been reported for *Chlorella*. Thus glycolic formation and excretion would not appear to arise from abnormal breakdown of ribulose diphosphate, but rather this is a normal pathway of metabolism from the pentose phosphates.

Gibbs: If one incubates pentose phosphate with (I hate to mention the word) cell-free preparations under anaerobic conditions, the pentose phosphate is converted to hexose phosphate. However, we found this summer if one does this under aerobic conditions the diose can be split off and converted to glycolic acid. We are attempting to purify this enzyme which takes a C_2 piece away from pentose phosphate and causes oxidation to form glycolic acid. Thus there is good evidence that glycolic acid can be formed directly from the so-called "active *glycolaldehyde*" piece of pentose phosphate.

Aronoff: It is interesting to note the excretion of glycolic acid during photosynthesis. However measurements on CO_2 exchange by roots, which presumably would be in a high atmosphere of CO_2, showed the presence of considerable glycolic acid in the root.

Tolbert: With roots the net exchange is that of bicarbonate or CO_2 arising from respiration and passing out of the root. According to our hypothesis of *bicarbonate-glycolate shift*, there should thus result an accumulation of glycolate inside the root cells.

M. B. Allen: Excretion of organic acids by *Chlamydomonas* might be of interest here, although this is not a transient phenomenon. In contrast to *Chlorella*, which excretes glycolic acid and then reabsorbs it, growing cultures of six species of *Chlamydomonas* have been found to accumulate soluble organic material in the culture medium (Allen, M. B., *Arch. Microbiol.*, in press). Formation of soluble products paralleled the growth of the alga and was favored by high light intensity and nitrate as nitrate source. Ten to forty per cent of the organic material synthesized by the algae appeared in the medium in soluble form.

Examination of the culture filtrates showed that the soluble organic material consisted partly of polysaccharide and partly of organic acids. The acids included glycolic, oxalic, and a keto acid which may be pyruvic.

Tolbert: For *Chlorella* the C^{14} labeled in the glycolate of the medium approaches a constant value after about 10 minutes. We are dealing with a dynamic exchange of bicarbonate and glycolate across the cell wall and not an excretion and continued accumulation in the medium of large amounts of glycolate.

Arnon: I don't know of any evidence for bicarbonate absorption by *Chlorella*. But work has been done with roots, and roots absorb very little bicarbonate. Is there any evidence that there is any bicarbonate absorption of the sort in algae?

Tolbert: Not exactly. The evidence just presented was that glycolate excretion occurred in medium at pH 5.5 or above and only in the presence of bicarbonate ion.

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2. *Photoreduction with Various Reductants*

Oxygen Evolution and Photoreduction by Adapted *Scenedesmus**

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The reaction steps on the oxygen-liberating side of the photosynthetic machinery are totally unknown. It was therefore of considerable interest when Gaffron reported that *Scenedesmus obliquus*, when suitably subjected to anaerobic incubation, became so adapted that now irradiation with weak light in an atmosphere of hydrogen and carbon dioxide resulted, not in the evolution of oxygen, but in the absorption of two hydrogen molecules for each carbon dioxide molecule consumed. In addition, he found that this adaptation allowed the algae to perform the oxyhydrogen reaction, in which, at sufficiently low oxygen tensions, the three gases· oxygen, hydrogen, and carbon dioxide, are normally consumed in the dark in the ratio 2:6:1.

He considered the possibility that the photochemical uptake of hydrogen and carbon dioxide by adapted *Scenedesmus* consisted of no more than photosynthesis and the oxyhydrogen reaction running concurrently (this suggestion was also made later by Larsen *et al.* (1)) according to the following equations:

(A) Photosynthesis

$$CO_2 + H_2O + \text{light} \rightarrow (CH_2O) + O_2$$

(B) Oxyhydrogen reaction

$$O_2 + 2H_2 \rightarrow 2H_2O$$

(C) Dark CO_2 reduction

$$qCO_2 + 2qH_2 \rightarrow q(CH_2O) + qH_2O$$

(D) $$(1 + q)CO_2 + 2(1 + q)H_2 + \text{light} \rightarrow (1 + q)(CH_2O) + (1 + q)H_2O$$

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Reaction (A) is simply ordinary photosynthesis as it is usually written. Reaction (B) is the oxyhydrogen reaction which supplies energy to accomplish the coupled carbon dioxide reduction indicated by reaction (C). The efficiency of the coupling between (B) and (C) is measured by q, which is normally $^1/_2$. The overall result of (A) + (B) + (C) is reaction (D) which differs from (E) below only by the additional gas consumption (q) due to the coupling of (B) to (C).

However, on the basis of the evidence available to him, Gaffron concluded that the light process he observed in adapted *Scenedesmus* was a true photoreduction, essentially like the process occurring in various photosynthetic bacteria in which molecular oxygen apparently does not play any role at all.

(E) $CO_2 + 2H_2 + \text{light} \rightarrow (CH_2O) + H_2O$

There were indications, however, in the work of Franck, Pringsheim, and Lad (2) that the situation was more complicated. At the suggestion of Gaffron, we have repeated and extended their experiments. The results indicate that although adapted algae can perform a true photoreduction they do not necessarily lose the capacity to evolve oxygen. True photoreduction with hydrogen and photosynthesis can exist side by side in adapted algae, and either may predominate depending upon conditions.

EXPERIMENTS WITH THE FRANCK-PRINGSHEIM OXYGEN APPARATUS

A careful comparison was made, using *Scenedesmus obliquus*, strain D_3, between the curve of oxygen evolution versus light intensity obtained in an atmosphere of hydrogen and that obtained in an atmosphere of nitrogen, both with 2% carbon dioxide. The rate of oxygen evolution was measured, as in the work of Franck *et al.* (2), by the method of phosphorescence quenching. Oxygen-free carrier gas passing over or through a cell suspension flushes away any oxygen that it may produce. After passing through a liquid nitrogen trap to remove moisture, it flows over a dye adsorbed on silica gel, whose phosphorescence is a measure of the amount of oxygen the carrier gas contains.

The algae were used at concentrations ranging from 0.0002% to 0.02%, and were flushed with oxygen-free carrier gas for a minimum

of 3 to 4 hours, often much longer, before beginning measurements. The partial pressure of oxygen in the carrier gas before entering the vessel was shown to be below 10^{-6} mm. Hg. There is no doubt that

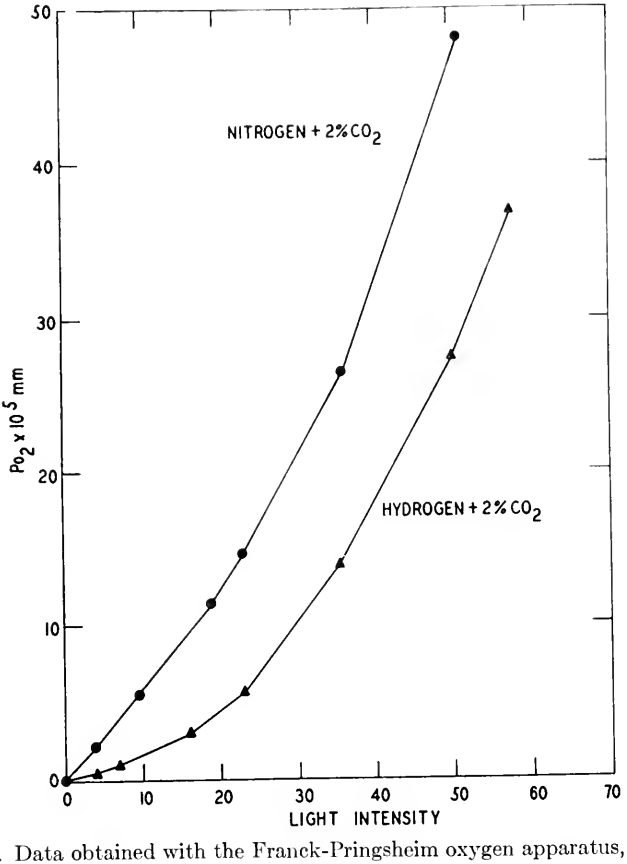

Fig. 1. Data obtained with the Franck-Pringsheim oxygen apparatus, comparing oxygen evolution from *Scenedesmus* D_3 incubated under hydrogen plus 2% carbon dioxide, and under nitrogen plus 2% carbon dioxide. Relative light intensity is plotted on the abscissa and oxygen pressure $\times 10^5$ millimeters of mercury, in the gas stream emerging from the reaction vessel, is plotted on the ordinate.

this partial pressure of oxygen is sufficiently low to permit adaptation.

Uniform illumination of all the cells was obtained by using green

light and only a relatively thin depth of very dilute suspension. The intensities were varied by interposing neutral screens in the beam, and were monitored with a Weston photocell and suitable galvanometer.

As is shown in Fig. 1, *Scenedesmus* evolves oxygen upon weak illumination, even after several hours of dark incubation in an atmosphere of pure hydrogen plus 2% carbon dioxide. However, at equal light intensities, the rate is always greater if the carrier gas is nitrogen rather than hydrogen. In obtaining these curves, the light intensity was increased stepwise from zero to the highest value shown on the graph. At this intensity the rate of oxygen evolution (in hydrogen plus carbon dioxide) corresponds approximately to one volume of oxygen per volume of algae per hour. With such low levels of oxygen production, the possibility of deadaptation is certainly remote.

The data of Fig. 1 clearly indicate that an active hydrogenase does not exclude the photosynthetic evolution of oxygen. However, because of the limitations in the apparatus used, it was not possible to estimate the extent of true photoreduction. It was, therefore, necessary to supplement these experiments with mass spectrometer and manometric ones.

THE MASS SPECTROMETER EXPERIMENTS

The instrument used in these experiments has been described by Brown *et al.* (3). Conventional Warburg vessels were attached to a male joint containing a very fine leak through which gas from the atmosphere above the algal suspension could enter the spectrometer analyzer tube at a very slow rate. In this way, there was continuous sampling of the gas phase in the Warburg vessel. At intervals of 1.8 minutes analyses were obtained for the four gases: oxygen 32, oxygen 34, carbon dioxide 44, and carbon dioxide 45.

Whereas in the Franck-Pringsheim apparatus only net oxygen production could be followed, in the mass spectrometer it was possible to measure oxygen and carbon dioxide consumption and production separately. Another desirable feature of the mass spectrometer was that it was possible to demonstrate adaptation during the course of the experiments by showing that carbon dioxide production is small (see column 2 of Table I) compared to the respiratory rate (0.6 to

0.8 cell volume per hour), although large amounts of oxygen are being consumed (in the oxyhydrogen reaction).

TABLE I. Mass Spectrometer Data on the Gas Exchange of Adapted *Scenedesmus* (Temperature is 20.6°C.)

(1) Experiment	(2) Respiration, cell vols./hr.	(3) O_2 production, cell vols./hr.	(4) CO_2/O_2	(5) % of ultraviolet inhibition of photosynthesis
080255-(2)	0.16	0.73	1.2	0
280454-(5)	0.14	1.8	1.0	30
280454-(7)	0.05	0.62	0.86	70
300454-(3)	0.29	3.0	0.78	0
300454-(5)	0.11	1.8	0.90	39
270155-(3)	0.13	0.84	1.2	63

A ratio indicative of the relative amounts of true photoreduction and photosynthesis which the algae are carrying on is that of carbon dioxide consumed photochemically to oxygen produced in the light. If there is only photosynthesis, this figure is close to 1 and if there is only true photoreduction, it is infinity. Combinations of the two processes will give intermediate values. The ratio has been calculated by assuming that the carbon dioxide fixation concomitant with the oxyhydrogen reaction occurs in the light as well as in the dark. The results are tabulated in column 4 of Table I.

In some of the experiments an ultraviolet treatment had just previously been administered to the algae. However, there is no reason to believe that this should invalidate the results. The data on carbon dioxide production indicate the cells were adapted, and previous work (4) has indicated that ultraviolet treatment stabilizes the adapted state.

The data obtained with the mass spectrometer and summarized in Table I give no reliable evidence for the existence of a true photoreduction in normal, adapted algae. They indicate that the photochemical activity of these algae is mainly, if not completely, photosynthetic. Nonetheless, since the measurements with the mass spectrometer were, of necessity, made in the presence of appreciable amounts of oxygen (0.04% to 0.24%), and oxygen tension could easily be an important variable in determining the extent of photoreduction, it was desirable to check for the presence of a true photo-

reduction under more completely anaerobic conditions. This was done with Warburg manometers, as is explained in the next section.

MANOMETRIC OBSERVATIONS

Larsen, Yocum, and van Niel (1) have pointed out that, if the photochemical consumption of hydrogen and carbon dioxide by normal adapted *Scenedesmus* consists of no more than photosynthesis and the oxyhydrogen reaction running concurrently, then this complex process should have a quantum yield for carbon dioxide uptake that is 50% higher than the quantum yield of photosynthesis alone. Another requirement of this kind of metabolism, however, is that the rate of carbon dioxide uptake not exceed the maximum rate of oxygen uptake in the oxyhydrogen reaction by more than 50%. Manometric evidence presented here indicates that this requirement is violated and that, therefore, a true photoreduction must exist in normal adapted cells.

Under conditions where oxygen tension is not limiting, the rate of oxygen uptake in the oxyhydrogen reaction at 28.3°C. by *Scenedesmus* D_3 is 3.6 cell volumes per hour. The maximum rate of carbon dioxide uptake in a process consisting of photosynthesis and oxyhydrogen reaction running concurrently is, therefore, 5.4 cell volumes per hour. However, it is possible manometrically to achieve rates of carbon dioxide uptake in the light that reach and even exceed 8 cell volumes per hour before deadaptation sets in. True photoreduction must, therefore, account for at least 30% of the photochemical carbon dioxide uptake under these conditions.

CONCLUSIONS

Our data require an interpretation more complex than is provided by heretofore prevailing views on the nature of the photochemical activity of adapted *Scenedesmus* under hydrogen and carbon dioxide. The present experiments make it clear that adapted *Scenedesmus* in the presence of hydrogen and carbon dioxide can, under certain conditions, perform photosynthesis at a rate which may even approach or reach that necessary to account for all its photochemical activity. Under these circumstances a correspondingly large part of the photochemical uptake of hydrogen and carbon dioxide by adapted *Scenedesmus* consists of the combination of photosynthesis and the

oxyhydrogen reaction running concurrently. On the other hand, under two of the limited number of circumstances explored in this and preceding papers, it is possible to demonstrate a true photoreduction. Gaffron had previously shown that in the presence of certain poisons, a true photoreduction occurs with oxygen production completely excluded. We were able to demonstrate a true photoreduction under the conditions obtaining in Warburg manometers, as discussed in the preceding section. These facts, of necessity, lead to the conclusion that the nature of the photochemical uptake of hydrogen and carbon dioxide by adapted *Scenedesmus* is variable, and depends on several factors which apparently control a sensitive balance between photoreduction and photosynthesis. Depending on the circumstances, there can be almost any proportion of photosynthesis and photoreduction in adapted algae, ranging from exclusive photoreduction to combinations of photoreduction, photosynthesis, and the oxyhydrogen reaction, to photosynthesis and the oxyhydrogen reaction running concurrently with photoreduction practically excluded.

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Photoreduction in *Ochromonas malhamensis**

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The chrysophyte flagellate *Ochromonas malhamensis* has been isolated and grown in pure culture by Pringsheim (1), and a synthetic medium for its cultivation has been devised by Hutner (2). Having thus become available as a laboratory subject, the physiology of *Ochromonas* has been studied by Meyers (3), Reazin (4), and Weis (5). It has been found that the organism fails to grow in the light unless a suitable organic substrate, such as glucose or acetate, is present. This observation suggested to Meyers (3) that *Ochromonas* contained insufficient chlorophyll to carry out photosynthesis at a rate required for growth and therefore multiplies by oxidative assimilation of an organic substrate. The ability of *Ochromonas* to grow under initially anaerobic conditions in the light renders this interpretation less plausible. An alternative interpretation is that the light-dependent metabolism of this alga may be in part a photoreduction, that is, a bacterial type of photosynthesis. This type of photosynthesis is characterized by the oxidation of an external hydrogen donor in place of oxygen evolution.

Photoreduction in algae was first observed by Gaffron (6), who found that hydrogen-adapted algae in dim light fixed CO_2 with the simultaneous uptake of hydrogen. Frenkel (7) has extended this observation to a wide variety of algae. However, in the instances studied photoreduction in algae was found only in resting thalli or cell suspensions; growth of algae by photoreduction was not observed. To investigate the possibility that *Ochromonas* might grow by photoreduction, the alga was grown in the continuous culture apparatus described by Benson *et al.* (8) on a synthetic medium. Two types of experiments were performed: manometric determina-

* Experiments were performed at the Brookhaven National Laboratory. Thanks are due to Dr. M. Gibbs, in whose laboratory the work was carried out, and to Dr. R. C. Fuller, whose culture apparatus was used.

tions of the activities of resting cell suspensions established the occurrence of photoreduction, and measurements of growth in a closed system showed that growth by photoreduction can occur.

Several attempts at inducing suspensions of *Ochromonas* to evolve or consume hydrogen failed but we do not consider these results conclusive. Isopropyl alcohol was found to be a suitable hydrogen donor for CO_2 fixation by *Ochromonas*. Its use was suggested by the work of Foster (9), who observed CO_2 fixation by *Rhodopseudomonas* with the concomitant light-dependent oxidation of isopropyl alcohol to acetone. The results summarized in Table I can be interpreted both in

TABLE I.* CO_2 Fixation by Resting Cell Suspension of *Ochromonas*

	Dark	Light, no addition	Light, 200 μM isopropyl alcohol
CO_2 (μM)	+10.5	+9.2	−15.2
O_2 (μM)	0	+1.2	+10.7
Acetone found (μM)	0.3	0.2	9.5

* 15-μl. cells per vessel, suspended in 2.0 ml. 0.05 *M* K-PO_4 buffer, pH 5.5. Atmosphere: Initially 95% N_2 + 5% CO_2. Illumination: 12,000 lux, incandescent light. Dark control wrapped in tin foil. Temperature: 30°C. Isopropyl alcohol added from sidearm at zero time. Time: 2 hours. Gas changes were determined as described in reference (10). Acetone was determined after distillation by condensation with salicylaldehyde.

a qualitative and in a quantitative fashion. Qualitatively, they show that a net fixation of CO_2 occurs in the light only in the presence of isopropyl alcohol. This alone suggests the occurrence of photoreduction. A quantitative interpretation of the experiment is complicated by our inability to decide what corrections should be applied for dark metabolism. Taking the figures at face value, we find that in the light without isopropyl alcohol 1.2 μM of oxygen is formed, presumably by photosynthesis, which therefore should correspond to the fixation of 1 μM of carbon dioxide. Yet we find a net evolution of 9.2 μM of carbon dioxide. Allowing for the carbon dioxide presumably fixed by photosynthesis, the total CO_2 evolution is 10.4 μM, which agrees well with the 10.5 μM of CO_2 given off by suspensions in the dark. In the light in the presence of isopropyl alcohol 10.7 μM of oxygen were given off, which should correspond to 10.7 μM of CO_2 fixed by photosynthesis, yet the net uptake of carbon dioxide observed is 15.2 μM, which leaves 4.5 μM of CO_2 fixed to be accounted

for. At the same time 9.5 μM of isopropyl alcohol have been oxidized to acetone, which is almost the stoichiometric amount required for the reduction of 4.5 μM of CO_2. These results are much too good to reflect the true course of events. The total amount of CO_2 fixed is probably greater than the net fixation observed since some fermentative CO_2 evolution probably also occurred. The amount of isopropyl alcohol oxidized is probably greater than 9.5 μM, since acetone can be further oxidized by *Ochromonas* and some of it undoubtedly was. But the results show quite clearly that CO_2 fixation by *Ochromonas* can be accounted for if we consider it to be the sum of both photoreduction and photosynthesis. These results merely extend the observation of photoreduction to another algal species, but Table II summarizes an experiment which provides evidence for growth of *Ochromonas* by photoreduction. Growing in the light on glucose in a closed system, *Ochromonas* consumed 29.6 mg. of carbon and formed cell material to the extent of 28.9 mg. of carbon. The ratio (mg.

TABLE II. Growth of *Ochromonas* on Two Substrates

	Glucose	Glycerol
mg. C of substrate used	29.6	22.0
mg. C of *Ochromonas* formed	28.9	28.5

Ochromonas was grown in closed flasks for 5 days at 30°C. Atmosphere: Initially 95% N_2 + 5% CO_2, no net formation of O_2 occurred. Illumination: *ca.* 1000 lux, fluorescent light. Glucose was determined by anthrone reagent, glycerol by periodate titration, and total carbon as $BaCO_3$ after $AgNO_3$ + K_2SO_5 combustion. No significant changes occurred in the dark.

C formed)/(mg. C used) = 0.98. A similar culture grown on glycerol consumed 22.0 mg. of carbon and formed cell material to the extent of 28.5 mg. of carbon, which gives a ratio (mg. C formed)/(mg. C used) = 1.29. In other words, when grown on glycerol, *Ochromonas* fixed more CO_2 than could be provided by the oxidation of glycerol. The ratio 1.29/0.98 = 1.31 corresponds approximately to the ratio between the number of hydrogens per carbon in glycerol to the number of hydrogens per carbon in glucose (2.66/2 = 1.33). Inasmuch as the amount of organic carbon formed during growth appears to be a function of the reduction level of the substrate, this experiment suggests that *Ochromonas* can grow by photoreduction. It is possible that this alga is limited in its ability to evolve oxygen.

Discussion

Frenkel: Is the assumption of the ratio of isopropanol used to CO_2 fixed dependent upon the product that is formed?

Vishniac: If you assume that carbohydrate was formed, then two moles of isopropyl alcohol would have to be oxidized to acetone to fix CO_2. If the end products are different then the ratio of isopropyl alcohol used to CO_2 fixed will change, but it would not change the general interpretation of this experiment.

Aronoff: Is it not possible that, even with glucose, you had CO_2 reduction?

Vishniac: Yes. We can assume, for instance, that all the organic carbon is formed by CO_2 fixation, but this goes along with oxidation of the glucose.

Aronoff: And having been at Brookhaven, were you not testing this with isotopes?

Vishniac: No, because I think that as far as these data are concerned we are dealing with net results and are not at a point where isotopes would help in the interpretation.

Frenkel: I was wondering if Dr. Gaffron wants to make some remarks about his experiments on the effect of glucose on photoreduction.

Gaffron: Glucose or glucose derivatives are capable of replacing molecular hydrogen as hydrogen donor during photoreduction in *Scenedesmus*. I think we have experiments which show that *Chlorella* might be trained to do this trick too, though for lack of a hydrogenase it cannot utilize free hydrogen. However, as yet I do not have a mass spectrograph. So we expect from Dr. Brown the next data on this.

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Manganese as a Cofactor in Photosynthetic Oxygen Evolution

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Manganese deficiency has been known for a long time to be a powerful inhibitor of photosynthesis in algae (1). Its effect on metabolism is unique in so far as only photosynthesis is affected and not respiration or chlorophyll formation (2,3). The inhibition of photosynthesis can be relieved within a short time by an addition of manganese (1–3); the degree of inhibition is independent of light intensity (2,3). The latter observation suggests a similarity between manganese deficiency and poisoning with hydroxylamine, a substance known to be a specific inhibitor of the oxygen-evolving process of photosynthesis (4,5). Therefore, Pirson suggested that manganese might perhaps be involved in the oxygen-liberating system of photosynthesis (2).

The process of photoreduction found in certain algae adapted to hydrogen (6) affords a possibility to test this hypothesis. In this reaction, the photochemical process and the reduction of CO_2 are presumably the same as in ordinary photosynthesis; only the evolution of oxygen is replaced by a reaction between its precursors and molecular hydrogen activated by hydrogenase (6). Therefore, an inhibitor specific for oxygen evolution in photosynthesis should not inhibit photoreduction and should, in addition, prevent the reversion of photoreduction to photosynthesis with evolution of oxygen which normally occurs at higher light intensities. This was shown to be the case in algae poisoned with hydroxylamine, *o*-phenanthroline, and phthiocol (4,7).

The green alga, *Ankistrodesmus braunii*, can be adapted to hydrogen within a few hours in an atmosphere of hydrogen + 4%

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CO_2. The light metabolism of this alga, grown with and without manganese, is shown in Fig. 1. Photosynthesis is inhibited to about one-fourth of the normal rate in manganese-deficient cells, the degree of inhibition being independent of light intensity. By contrast, photoreduction is not at all inhibited by manganese deficiency; its rate in

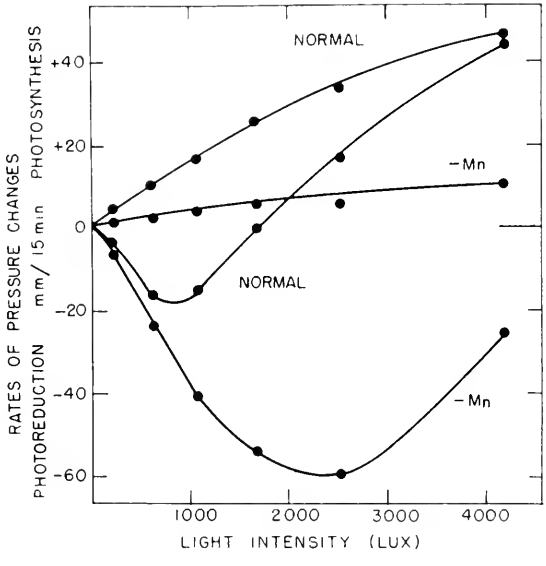


Fig. 1. Rates of photosynthesis and photoreduction at various light intensities in normal and in manganese-deficient *Ankistrodesmus braunii*. Manometric readings correspond to evolution of oxygen (corrected for respiration) in photosynthesis and to absorption of hydrogen and carbon dioxide in photoreduction. Experimental conditions: Warburg No. 9 buffer, pH 9, gas phase air, for photosynthesis; 0.02 *M* phosphate buffer, pH 6.5, gas phase 96% H_2 + 4% CO_2, purified by means of a "Deoxo" cartridge, for photoreduction; temperature 25°C., 10 mm.^{3} cells (3 mg. dry weight) per vessel. Preceding dark period: 4 hours; periods of 15 minutes for each light intensity.

the light-limited range is even somewhat higher in the deficient cells (*cf.* 8). At higher light intensities extremely high rates of photoreduction are observed with the deficient algae, whereas in normal organisms deadaptation and evolution of oxygen occur. Thus the action of manganese deficiency strongly resembles the effect of hydroxylamine, *o*-phenanthroline, and phthiocol (4,7). At high concentrations these poisons, however, are more efficient in stabilizing

photoreduction at very high light intensities than is manganese deficiency. This is quite understandable since the concentration of poison required inhibits photosynthesis completely, whereas manganese deficiency does not. Other differences between poisoned and deficient algae are the following: (*1*) The above-mentioned poisons, if added prior to adaptation, will inhibit the process of adaptation to hydrogen (4,7). With manganese-deficient algae, no such inhibition of adaptation is observed. (*2*) High concentrations of poisons reduce the quantum yield of photoreduction (i.e., the rate at low light intensities) up to 50% (4,7). With increasing strength of manganese deficiency, however, up to an age of five weeks for the deficient cultures, the rate of photoreduction in the light-limited range remains absolutely constant. At the same time, the protection of photoreduction against deadaptation at higher light intensities increases steadily. (By contrast, the yield of photosynthesis drops to about one-fourth during the first 7 to 10 days of deficiency.) Later the manganese-deficient cells become chlorotic, the yield of photoreduction decreases, and the algae deteriorate rapidly.

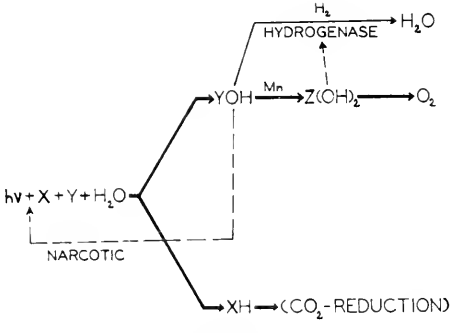
An addition of manganese to deficient cells results under aerobic conditions in the well-known increase in photosynthesis (1–3); with adapted algae, however, a decrease of photoreduction occurs because deadaptation is accelerated and starts at lower light intensities.

In the light-limited range, the amounts of CO_2 reduced per unit of time are approximately the same for normal algae in photosynthesis and photoreduction and for manganese-deficient algae in photoreduction. These deficient algae, however, can reduce only one-fourth as much CO_2 in aerobic photosynthesis.

Studies performed with algae deficient in phosphate and in iron (*cf.* 2) show that the effect of manganese is quite specific for this ion. Phosphate deficiency inhibits photosynthesis and photoreduction to about the same degree; the percentage inhibition of photosynthesis increases with increasing light intensity. The effect of phosphorus deficiency resembles the action of dinitrophenol on photoreduction as observed by Gaffron with *Scenedesmus* D_3 (4). Iron deficiency inhibits photoreduction much more strongly than photosynthesis. In this connection it should be mentioned that photoreduction has been found to be very sensitive toward cyanide and carbon monoxide (4).

Previously it was suggested (3,9) that manganese might be primarily involved in the basic photochemical reaction of photosynthesis. However, from our results it follows that its main role in photosynthesis must be concerned with oxygen evolution, the only process of photosynthesis which is not needed in photoreduction. Also the results of Gerretsen (10) with chloroplasts from higher plants suggested the importance of manganese for the formation of peroxides. Recently Kenten and Mann (11) have demonstrated a light-dependent oxidation of manganese in chloroplast preparations and have discussed its possible role in photosynthesis. It might well be that there is an additional and perhaps smaller requirement for manganese in CO_2 reduction, as suggested by the work of Arnon *et al.* (12) with chloroplasts. This, however, could be detected only at saturating light intensities which cannot be reached in photoreduction because of deadaptation.

The hypothetical scheme I might serve to explain the observation that the inhibition of photosynthesis by manganese deficiency is



Scheme I.

independent of light intensity, although an enzymatic reaction, namely, oxygen evolution, is affected primarily. We assume that manganese is specifically involved in the transformation of the first oxidized product of photosynthesis, YOH, to a peroxide, $Z(OH)_2$, which eventually gives off oxygen (*cf.* 6). Normally, in the presence of manganese in algae not adapted to hydrogen, all the YOH formed will be disposed of to evolve oxygen. In adapted algae, however, most of the YOH reacts with hydrogen activated by hydrogenase to

form water; under these conditions only a comparatively small fraction of the YOH goes via $Z(OH)_2$ to O_2 (13). With increasing light intensity, more $Z(OH)_2$ is formed, which eventually oxidizes the hydrogenase and thereby causes deadaptation to normal photosynthesis with evolution of oxygen. In the case of manganese deficiency, the formation of $Z(OH)_2$ is inhibited; thus deadaptation of photoreduction is prevented or at least considerably delayed, and all the YOH formed will be disposed of by the hydrogenase reaction, thereby avoiding the step requiring manganese in the evolution of oxygen. In unadapted algae, however, this possibility does not exist. Therefore, the YOH may react back causing chemiluminescence (14) or oxidize cellular substances. This latter reaction may lead to the formation of a "narcotic" (15), which, in turn, will inhibit the photochemical reaction, thus reducing the rate of photosynthesis at all light intensities.

Discussion

Aronoff: How many generations occur after starting a manganese-deficient culture?

Kessler: Growth is not completely inhibited by manganese deficiency. It is only retarded, but I don't know how many generations there are.

Jacobs: Can the inhibition of photosynthesis be relieved by the addition of manganese?

Kessler: Yes. If one adds manganese to deficient cells, the rate of photosynthesis will go up to the normal level. The speed of recovery depends upon the age of the deficient culture. In the beginning it goes very fast. With strong deficiency, however, the rate of photosynthesis goes up to the normal level within about 10 hours or so. But if one adds manganese in photoreduction, then the addition of manganese will inhibit photoreduction and will accelerate deadaptation.

Arnon: On this point of time that Dr. Kessler has answered, I would like to say that we are able to confirm that under certain conditions the minus manganese cells can restore their full photosynthetic rate equal to that of the plus-manganese cells within 20 minutes after the addition of manganese. This is far less time than required for any new cell division or even formation of a protein.

Rosenberg: I wonder if you have any data on the concentration of manganese in chloroplasts in normal cells and in deficient cells.

Kessler: No, I don't have any analytical data on the manganese content of the cells. I also haven't done any experiments on the Hill reaction, but I know from unpublished work of Clendenning that the Hill reaction is also inhibited by lack of manganese, and that its rate can be increased by the addition of manganese.

Also Gerretsen has shown that manganese is somehow involved in peroxide formation in isolated chloroplasts. However, it is not quite clear from his data what his results have to do with normal photosynthesis. He worked with isolated chloroplasts in the absence of Hill reagents, measured the oxidation-reduction

potential, and found that it changed in a very marked way upon the addition of manganese, suggesting that peroxide is formed in the presence of manganese but not in its absence.

Spikes: In isolated, well-washed chloroplasts we can find practically no manganese.

Kessler: Probably the chloroplasts of nondeficient plants contain an amount of manganese sufficient for the maximum rate of the Hill reaction.

Spikes: If it is there, it must be in a very tightly bound form, because 20 or 30 washings of isolated chloroplasts do not result in any marked decrease in the rate of the Hill reaction.

Arnon: I think that the evidence in support of the role of manganese in oxygen evolution is quite substantial. However, this would not necessarily exclude the participation of manganese in CO_2 fixation. Aren't we assuming that the CO_2 metabolism in photoreduction is similar to that in photosynthesis. I take it that there are no chemical data to tell us that they are in fact similar.

James Smith: Long ago, when we treated sunflower leaves with carbon dioxide we could demonstrate that manganese was one of the carbon dioxide acceptors. When we treated leaves with carbon dioxide in order to see what compounds were soluble we could extract manganese, magnesium, and calcium, and we could demonstrate by radiocarbon experiments that the manganese certainly entered into this and was part of the reservoir for the carbon dioxide uptake. I don't know how this fits into your picture, but it may have something to do with it.

Arnon: The conclusion I was leading up to was this and this again is an assumption: If the CO_2 fixation pattern during photoreduction were altered by manganese deficiency in such a way that light would not be able to accomplish deadaptation, this would still be compatible with the results that you presented.

Now let me add, as I stated earlier, that we ourselves have also found that oxygen evolution is influenced by manganese deficiency and that the effect is reversible, but at the moment the other possibility should be kept open at least as a subsidiary point.

Jacobs: Can the requirement for manganese be replaced by higher levels of magnesium, cobalt, or nickel?

Kessler: Cobalt and nickel are not present in our culture medium except for those impurities which are always introduced with iron and zinc, and the other nutrient components, but quite a high concentration of magnesium is present in both the normal and the deficient medium.

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3. *Reduction of Various Oxidants*

Contributions to the Problem of Photochemical Nitrate Reduction

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It has been known for a long time that light has a strong accelerating influence upon nitrate reduction in green plants (1–3). In spite of many studies, however, the reason for this light effect is still obscure. Several explanations have been suggested: (*1*) The mechanism of nitrate reduction might be essentially the same in the light and in the dark, the higher rate observed in the light being due to a larger supply of carbohydrates produced by photosynthesis. (*2*) The influence of light might be an indirect one, due to special products of photosynthesis reacting with the nitrate (4). (*3*) A chlorophyll-sensitized oxidation by nitrate of organic compounds (5). (*4*) A photochemical reduction of pyridine nucleotides with subsequent transfer of the hydrogen to nitrate (6). (*5*) A photochemical reduction of nitrate with nitrate acting instead of CO_2 as the hydrogen acceptor in photosynthesis (7–9).

A truly photochemical reduction of nitrate, if it exists at all, should easily be demonstrated with algae in the light in the absence of CO_2. Experiments of this kind, carried out under pure nitrogen in order to prevent respiration and photooxidations, would be an obvious way to exclude the first two possibilities mentioned above, namely, the occurrence of a nitrate reduction bound to respiration or indirectly accelerated by carbon intermediates of photosynthesis in the light. All experiments performed thus far that have been interpreted as supporting a "photochemical reduction of nitrate," however, have been made in the presence of CO_2. On the other hand, all attempts to

* This work was performed during the tenure of a fellowship awarded by the National Academy of Sciences and International Cooperation Administration. Permanent address: Botanisches Institut, Universität Marburg, Marburg/Lahn, Germany.

demonstrate a really significant reduction of nitrate with algae in the absence of CO_2 have been unsuccessful (10–12). It is only when glucose has been added in the light that after several hours a strong evolution of oxygen due to nitrate reduction occurs (12). Also under the extreme conditions of the "nitrate mixture" (0.1 *M* $NaNO_3$ + 0.01 *M* HNO_3), a two- to threefold increase in reduction in the light without CO_2 over the rate of the dark reaction has been observed (2). The small evolution of oxygen in the presence of nitrate, reported by Kok (11), can hardly be regarded as convincing proof of the existence of a photochemical nitrate reduction. All these observations point to an indirect role of light via the formation of photosynthetic carbon compounds rather than to the existence of a direct photochemical reduction of nitrate.

Our earlier observation that light very much accelerates the reduction of nitrite, the first intermediate of nitrate reduction in algae (13,14), formed the basis of our new approach to this problem. The nitrite accumulated by the green alga, *Ankistrodesmus braunii*, in an acid culture medium (pH<4) in the dark rapidly disappeared upon illumination (13). In these experiments, performed in the presence of CO_2, glucose was not an effective substitute for light. A similar influence of light on the reduction of nitrite by diatoms has been observed by Harvey (15).

The present experiments compare the rate of nitrate reduction to that of nitrite reduction in the light in the absence of CO_2 (Fig. 1). Upon addition of nitrate to a suspension of *Ankistrodesmus braunii* in N-free culture medium, only a very small amount of oxygen is evolved. This result agrees very well with the previous observations of other authors (10–12). The rate of nitrate reduction under these conditions is only about 10% of that observed in the light in the presence of CO_2 or of glucose (12). On the other hand, an addition of nitrite leads to a strong evolution of oxygen (*cf.* 16). For every mole of nitrite reduced, 1.5 moles of oxygen are evolved. In contrast to the reduction of nitrite in the dark, the rate of this reaction in the light considerably increases with increasing hydrogen ion concentration within the physiological range. The rate of oxygen evolution with nitrite is comparable to that in the quinone Hill reaction in whole *Ankistrodesmus* cells.

Furthermore, we have studied the influence of low temperatures on the reduction of nitrite under different conditions in the light and

in the dark. The results are shown in Table I. A decrease in temperature from 15°C. to 4°C. inhibits nitrite reduction in the dark and in the light in the presence of CO_2 by about 50%. In the light with-

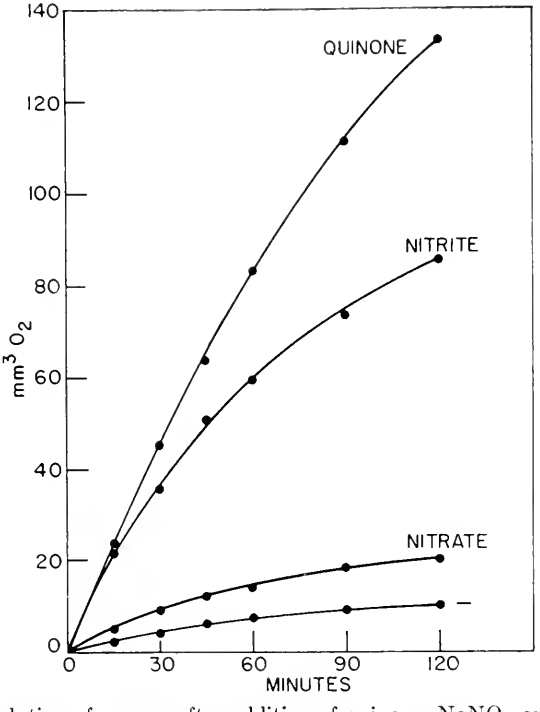

Fig. 1. Evolution of oxygen after addition of quinone, $NaNO_2$, and KNO_3 (no addition as control) to *Ankistrodesmus braunii* suspended in N-free culture medium (pH 6.5) in the absence of CO_2. Gas phase N_2 (KOH in the center well); light intensity 13,000 lux; temperature 15°C.; 21 mm.³ cells (6.5 mg. dry weight) per vessel. Concentrations of oxidants: Quinone 3.3×10^{-3} mole per liter; $NaNO_2$ 1.1×10^{-3} mole per liter; KNO_3 8.3×10^{-4} mole per liter. Theoretical yield: 179 mm.³·O_2 in each case. Pretreatment of the algae: 4 hours in N-free medium at moderate light intensity (4000 lux); gas phase air.

out CO_2, however, nitrite reduction is decreased by only 15%. The favorable influence of CO_2 on the reduction of nitrite almost disappears at 4°C. Light under anaerobic conditions in the absence of CO_2 is much more effective in nitrite reduction than is respiration in the dark. This especially holds true at 4°C., where the rates of enzymatic reactions are strongly decreased.

TABLE I. Reduction of Nitrite (moles $\times 10^{-8}$) by 25 mm.^{3} *Ankistrodesmus* Cells in 1 Hour at pH 4.5. $NaNO_2$ (5.5×10^{-4} Moles per Liter) Added to Algae in N-free Culture Medium. Concentration of Nitrite Measured Colorimetrically. Experimental Conditions Otherwise as Indicated in Fig. 1

	Light		Dark	
	N_2 + 4% CO_2	N_2	Air	N_2
15°C.	212	108	40	20
4°C.	110	90	20	8

These findings indicate the important role of photochemical processes in the reduction of nitrite. It should be stressed, however, that there are some marked differences between the reduction of nitrite and that of an ordinary Hill reagent. As shown in Fig. 1, the rate of nitrite reduction decreases faster than that of quinone reduction. Moreover, a second addition of quinone immediately results again in a strong evolution of oxygen, whereas after a second addition of nitrite only a small increase of oxygen production is observed. The rate of nitrite reduction is apparently determined by the supply of carbon compounds necessary for the further assimilation of the products of its reduction. In addition, isolated chloroplasts of higher plants, which readily reduce quinone and other Hill reagents, are unable to reduce nitrite (6,13), just as they do not reduce nitrate (17) unless nitrate reductase and triphosphopyridine nucleotide are added (6).

From these results we conclude that the mechanisms of nitrate and nitrite reduction by algae in the light are essentially different. The first step of nitrate reduction, nitrate → nitrite, which goes on rapidly only in the presence of CO_2 or of glucose, seems to be indirectly accelerated by light via the photosynthetic formation of carbon compounds. The further reduction of nitrite, however, seems to be more closely connected to photochemical processes. Recently Vanecko and Frear (18) have obtained results with higher plants that point in the same direction.

Addendum

Recent experiments have shown that the reduction of nitrite in the light without CO_2 saturates at low light intensity (approximately 750 lux). Furthermore, this reaction is less sensitive toward dinitrophenol (DNP) than is the aerobic reduction of nitrite in the dark

(19). A concentration of 2×10^{-4} molar DNP at pH 6.3 decreases nitrite reduction in the dark by about 85%, whereas the reaction in the light is inhibited by only 15%.

It is of interest in this connection that (*1*) glucose assimilation in the light, a process dependent upon ATP formed by photosynthetic phosphorylation, attains saturation at low light intensity (20); (*2*) phosphorylation in the light seems to be less sensitive toward DNP than is phosphorylation coupled to respiration (21); (*3*) ATP formation in the light is only slightly decreased at low temperature (22). In these three properties photosynthetic phosphorylation (23) and nitrite reduction in the light show a striking similarity.

Since the reduction of nitrite to ammonia, in contrast to the reaction nitrate → nitrite, seems to require ATP (19), the effect of light on nitrite reduction might be due to a supply of energy-rich phosphate bonds by means of photosynthetic phosphorylation. This possibility has recently been discussed also by Stoy (24).

Discussion

Pirson: You have shown that it is possible to separate photosynthesis from nitrite reduction by lower temperatures. Would nitrite reduction be a pure photochemical process without any enzymes?

Kessler: No, I think that possibility is completely excluded. If this were the case, one should assume that cells in the light with no CO_2 should reduce very large amounts of nitrite without any decrease in rate of reduction.

Pirson: You must assume that the temperature coefficients of the photosynthetic enzymes and those involved in nitrite reduction are completely different.

M. B. Allen: I would like to ask Dr. Kessler if he has any idea how the carbohydrate might affect nitrate reduction.

Kessler: It might be that it acts as a hydrogen donor, that the reduction of nitrate is somehow connected with the oxidation of carbohydrate. This is probably the simplest explanation.

M. B. Allen: I find this result rather difficult to reconcile with the experiment of van Niel and myself and the demonstration of Evans and Nason that one can reduce nucleotides with chloroplasts, add these to nitrate-reducing enzymes, and get nitrate reduction.

Kessler: The results of Evans and Nason on photochemical nitrate reduction are, according to my opinion, not conclusive. They added to the chloroplasts nitrate reductase and TPN. However, it is not necessarily so that these substances must also be involved in light nitrate reduction in the living cell. And the fact remains that nitrate is not reduced by our algae in the absence of CO_2, whereas nitrite is. If one assumes this Evans-Nason type of reduction, then one should expect that nitrate will be readily reduced by the cells, but this does not occur. Only nitrite will be reduced, which is not reduced by the Evans-Nason system.

Tolbert: How many plant tissues have you tried?

Kessler: These experiments have been done only with *Ankistrodesmus* cells.

Myers: Following such nitrite reduction as you observed, do the cells remain viable?

Kessler: Yes, they do.

Kamen: Nitrite is very closely associated with some hemoproteins, and it may be that there may be an inhibition on this ground, if the nitrate reduction of this particular organism goes through a cytochrome. Verhoeven and Takeda got a preparation from *Pseudomonas aeruginosa* which contained a flavin protein that activated the reduction of both nitrate and nitrite by reduced cytochrome *c*. This means very likely, although not necessarily, that there may be some cytochrome involved in the reduction of the nitrite in this particular organism.

Gibbs: You measured only nitrite reduction. Did you determine whether some of the nitrite was going back to nitrate?

Kessler: I have made tests for nitrate after nitrite reduction. They were always negative.

M. B. Allen: I would like to say that no reduction of nitrate in the absence of CO_2 is not necessarily a general phenomenon in algae. We have carried out a very appreciable reduction of nitrate with *Chlorella* in the absence of CO_2. In fact, the amount of oxygen produced from nitrate when CO_2 was absent was comparable to the excess of oxygen over CO_2 which we obtained in the presence of nitrate. Since we know that algal metabolism is a rather plastic thing, it seems to me not unreasonable that we could get conditions in which nitrate reduction and carbohydrate metabolism were mixed up together.

Kessler: There might be differences between different strains. But, as far as nitrate reduction in the light is concerned, the results obtained with *Ankistrodesmus* are almost identical with those of Davis with *Chlorella*. In both cases no appreciable reduction of nitrate occurs in the light unless CO_2 or glucose is present.

M. B. Allen: But we can take one *Chlorella* strain under different conditions and it will change its habits. The point which I wish to make is that the interrelation between carbon metabolism and nitrate reduction may depend on how the alga has been cultivated.

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Certain Effects of Ascorbic Acid on the Reduction of Oxygen in Chloroplast Preparations*

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Some years ago Alan Mehler discovered that oxygen should be added to the growing list of Hill oxidants which are effective in promoting photochemical oxygen production by chloroplast preparations. Mehler observed a light-dependent oxygen consumption in the presence of a hydrogen peroxide trap (ethanol plus a large excess of catalase). He reasoned that oxygen was being reduced to peroxide by a photochemically generated reductant. This reaction sequence accounts for oxygen consumption in accordance with the stoichiometry of the following scheme:

(A) $4H_2O \rightarrow 4[H] + 4[OH]$
(B) $4[OH] \rightarrow 2H_2O + O_2$
(C) $2O_2 + 4[H] \rightarrow 2H_2O_2$
(D) $2C_2H_5OH + 2H_2O_2 \rightarrow 2CH_3CHO + 4H_2O$

$O_2 + 2C_2H_5OH \rightarrow 2CH_3CHO + 2H_2O$

The net uptake of oxygen is thus the consequence of two molecules consumed for every one produced. Mehler was able to confirm this two-way traffic in oxygen metabolism by the use of tracer oxygen monitored with a mass spectrometer.

When Mehler described the properties of this reaction, he noted that the rate was considerably enhanced if the chloroplast preparation had previously been allowed to reduce quinone. One can speak of a stimulated as well as an unstimulated Mehler reaction and, since a number of substances are capable of effecting such a stimulation, it is meaningful to distinguish between, say, a quinone-stimulated and a manganese-stimulated Mehler reaction.

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In the absence of an ethanol-catalase trap for the hydrogen peroxide, a negligibly small oxygen metabolism by chloroplast preparations is observed manometrically either in light or dark. With tracer oxygen, however, an exchange of oxygen is found to occur in light in 1:1 ratio. There is some evidence that this production balanced by consumption is the consequence of catalytic dismutation of the hydrogen peroxide by endogenous catalase; in other words reaction (E) $2H_2O_2 \rightarrow 2H_2O + O_2$ replaces (D) in the previous scheme to account for the light dependent oxygen exchange. We have found that this exchange reaction, detectable only with isotopically enriched oxygen, also is subject to stimulation by a previous quinone reduction in the light. Therefore we can study the properties of either the stimulated or the unstimulated exchange reaction.

Among the substances which Mehler tested for their possible effect on the quinone-stimulated Mehler reaction, ascorbic acid was especially interesting in that its enhancement of the reaction rate was not influenced by the presence of metabolic poisons which have been considered relatively specific for the photosynthetic partial reaction of oxygen liberation. Mehler suggested that one or both of the steps of ascorbic acid oxidation in the light might utilize an oxygen precursor from the chloroplasts rather than molecular oxygen—the stimulation of gas uptake being therefore due partly or entirely to decreased production instead of increased consumption of oxygen. It is of some interest to investigate further this suggestion and to determine whether ascorbic acid is indeed a reagent which can attack a reactant in that part of the photosynthetic reaction sequence lying between the oxidized photolysis product and molecular oxygen.

To test Mehler's hypothesis we have employed tracer oxygen and a recording mass spectrometer in a fashion described in previous communications. The rates of simultaneous production and consumption of oxygen were computed from the rates of metabolism of the oxygen isotopes when the oxygen of the gas phase in the experimental vessel was enriched with tracer of mass 34. Chloroplasts of "Pokeweed" (*Phytolacca decandra* L.) have been used throughout. The experiments are still in progress but enough has been learned to make worth-while the presentation of some of our results which pertain to the manner in which ascorbic acid affects the oxygen metabolism of illuminated chloroplast preparations.

Figure 1 shows the results of six experiments on the quinone-stimulated Mehler reaction. Oxygen evolution rates are plotted above and oxygen consumption rates below the base line. In each experiment the first period is a dark control. The second period shows the oxygen production rate of a quinone-Hill reaction. Thereafter the preparation (now quinone-stimulated, of course) exhibits a Mehler reaction in which oxygen uptake proceeds at twice the rate of oxygen evolution. Then, upon addition of ascorbic acid, the influence of this substance on the individual production and consumption components of the stimulated Mehler reaction is observed. The final period is

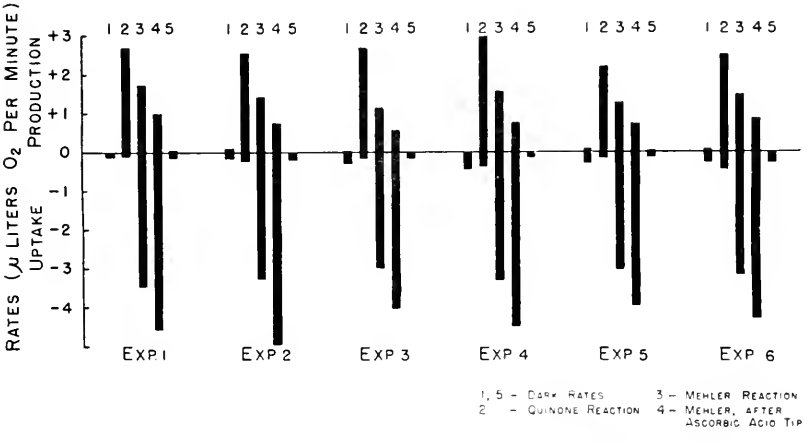

Fig. 1. Effect of ascorbic acid on quinone-stimulated Mehler reaction.

again dark. It is apparent that the stimulation of net oxygen consumption brought about by the ascorbic acid is attributable to both increased consumption and decreased production.

At present our interpretation of these results lies along the lines of Mehler's original suggestion that ascorbic acid oxidation in the light utilizes an oxygen precursor. In terms of the detailed reaction steps below which have been postulated to account for the oxygen metabolism of the chloroplasts, we note that at all but the lowest light intensities (at which the photolysis reaction (F) becomes rate-limiting) the back reaction (G) may be important. When quinone is being reduced, its reaction (H) must effectively compete with the back reaction (G) so that oxygen evolution (J) is enhanced. When

(F) $2H_2O \xrightarrow{h\nu} 2[H] + 2[OH]$

(G) $2[H] + 2[OH] \rightarrow 2H_2O$

(H) quinone $+ 2[H] \rightarrow$ hydroquinone

(J) $2[OH] \rightarrow {}^1/_2O_2 + H_2O$

(K) $2[H] + O_2 \rightarrow H_2O_2$

(M) ascorbic acid $+ 2[OH] \rightarrow 2H_2O +$ dehydroascorbic acid

the quinone has all been reduced, the first step in the Mehler reaction (K) may begin. We may think of the back reaction (G) as competing in a sense with oxygen production (J), on the one hand, and with either quinone (H) or oxygen reduction (K) on the other.

We have generally found that the oxygen production rate via the Mehler reaction is appreciably lower—often much lower—than that of the quinone-Hill reaction. Therefore reaction (K) does not compete with (G) as well as (H) does so that, during the Mehler reaction, a back reaction of the photolysis products must occur at a rate which is not negligible. When ascorbic acid is introduced into the system, reaction (M) with the photolytic oxidation product may be postulated, and this of course could compete with oxygen evolution (J), giving the decreased oxygen production which was observed. If we suggest further that the ascorbic acid reaction (M) competes also with the back reaction (G), then the ascorbic acid, serving as a trap for [OH], would reduce the rate of the back reaction (G) as well as that of the oxygen evolution (J). Slowing the back reaction must inevitably promote oxygen uptake in reaction (K); thus ascorbic acid has a dual effect of reducing oxygen evolution and enhancing oxygen consumption, as the experimental results in Fig. 1 testify. At the moment we favor this explanation of the ascorbic acid stimulation of the Mehler reaction, and we are studying other aspects of the system in an effort to apply additional tests of the proposed mechanism.

It is interesting to note that the Mehler reaction was discovered when Alan Mehler attempted to identify as hydrogen peroxide the oxidation product [OH] of the chloroplast reaction. Although this attempt failed because he observed no reaction of his reagent (catalase plus ethanol) with an oxygen precursor, he did perhaps discover that another substance, ascorbic acid, could play the role of reactant with the [OH]. If our provisional interpretation is correct, the ascorbic acid acts early enough in the reaction sequence leading

to oxygen evolution to influence the back reaction. It is of course still speculative whether or not naturally occurring ascorbic acid functions in this way.

Discussion

Lumry: I have two questions. What is known about oxygen reduction unstimulated by quinone? Has anyone been able to reverse the stimulation produced by quinone?

Brown: When studied manometrically the rate of net oxygen consumption in the quinone-stimulated Mehler reaction usually is more than twice that of the unstimulated reaction. The enhancement factor varies with different chloroplast preparations and may even be zero. With a given batch of "cooperative" chloroplasts results are consistent, however. The effect of quinone concentration on the rate of the subsequent (stimulated) Mehler reaction after the quinone reduction is over has been investigated, and this relation is a roughly hyperbolic curve saturating at about 2×10^{-3} molar. We originally thought that the quinone-stimulated Mehler reaction proceeded exactly twice as fast as the unstimulated when enough quinone was present to saturate the stimulation effect; it now seems that the factor usually is above two. Also the effect of ascorbic acid on the unstimulated Mehler reaction has been studied, and it was found that a slight but consistent stimulation of oxygen production occurred in that case.

In answer to your second question, we have not observed a reversal of the quinone stimulation on the Mehler reaction. Depending on experimental conditions the rates of either stimulated or unstimulated Mehler reactions decline with time—say 10% or no more than 20% in a couple of hours. Within limits of manometric measurement no appreciable return to the unstimulated reaction rate has been observed.

Lumry: Quinone and perhaps other Hill oxidants play a very strange role in several chloroplast reactions. We have noticed that the change-over from one type of Hill reaction to another, produced by increasing the oxidant concentration, takes place in just the same range as the stimulating effect. It is also in this region that fluorescence quenching by Hill oxidants begins to appear. The parallelism between effects of quinone on the Mehler reaction and on the Hill reaction may ultimately prove to be less close than it now appears, but certainly the entire matter is worthy of much more detailed investigation. It would, for example, be interesting to see if other Hill oxidants can stimulate the Mehler reaction, since all we have investigated have similar effects on the Hill reaction. Perhaps one might find a new way to get manganese ion into photosynthesis in such studies. The latter substance, like quinone, presents some highly intriguing possibilities but neither can be said to be well studied.

Brown: Stimulation of the Mehler reaction by Mn^{++} has been studied also. Here the effect is more striking than with quinone. Somewhat higher stimulation factors are observed and the effect saturates at about 0.5×10^{-3} molar.

Tamiya: Does oxygen compete with quinone in the Hill reaction?

Brown: Everyone assumes that they compete. Initially you start out with a concentration of quinone high relative to that of oxygen and you end up with the

quinone all used up and plenty of oxygen present. Mehler and I have published data showing with tracer oxygen that, as long as quinone is present, you observe no oxygen consumption; tracer oxygen uptake begins only at the end of the quinone reaction.

Frenkel: Has vitamin K worked?

Brown: It does not improve the situation. It was used in Kamen's system. He may have some comments about that. We could not confirm the ameliorating effect of the vitamin K. It was not necessary to add it to our system in order to get the maximum ascorbic acid effect. So in the work reported it was not included.

Kamen: Our experience relates only to *Chromatium*. Dr. Newton and I have not been able to get any effect of increased efficiency with menadione in carrying out phosphorylation in light. Moreover, we cannot find any evidence whatever for any vitamin K either in *Chromatium* or in the chromatophore. I don't know about *Rhodospirillum rubrum*.

As far as the reduction of oxygen is concerned, Dr. Vernon and I did a large amount of work on that and our experience was something, it seems to me, very close to that of Wessels', in that we found that we could not oxidize ascorbic acid unless we added the hydroquinone or the quinone as a carrier.

Duysens: Wessels recently gave an alternative explanation of the effect of ascorbate and quinone. To support his explanation he did the following experiment: He used a chlorophyll solution in ethanol and added 2,6-dichlorophenol-indophenol and ascorbic acid and he got an uptake of oxygen and an oxidation of ascorbic acid. This experiment indicates that the light-induced oxidation of ascorbic acid in chloroplast suspensions is quite different from the "normal" Hill reaction.

Arnon: It seems to me that the point made by Dr. Duysens that you can get a similar effect with chlorophyll solution as distinguished from chloroplasts raises a question about the physiological significance of this effect.

Duysens: The ascorbic acid oxidation in chloroplast suspensions is not affected by various metal poisons which are inhibitors of the "normal" Hill reaction which indicates that it is a nonenzymatic photochemical reaction between chlorophyll and oxygen.

Brown: But the mechanism of the ascorbic acid reaction which Mehler tentatively proposed is such as to predict that the process should be less sensitive than the Hill reaction to inhibitors of oxygen evolution. In other words, this result is to be expected on the basis of Wessels' photosensitized oxidation explanation, which you favor, as well as from the standpoint that oxygen is consumed via Mehler's mechanism, which I prefer. We seem to be using the same data to support different mechanisms.

Vernon: What Miss Habermann and Dr. Brown have shown is the coupled reaction. The ascorbic acid stimulates both parts—both the uptake and evolution of oxygen—which means that it is not strictly a photochemical oxidation of the ascorbic acid. You are stimulating two parts of the system, and I think that it is a distinction between chlorophyll in solution and chloroplasts.

Brown: Ascorbic acid does stimulate both oxygen production and consumption in the case of an unstimulated Mehler reaction. However, in the case of the quinone-stimulated Mehler system, ascorbic acid also increases uptake but decreases production of oxygen.

4. *Reactions in Chloroplasts and Cell Extracts*

Some Features of the Chloroplast Reaction

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HYDROGEN ACCEPTORS FOR THE CHLOROPLAST REACTION; SOME PROPERTIES OF A NATURAL HYDROGEN ACCEPTOR

Chloroplast preparations liberate oxygen from water in the light in the presence of a suitable hydrogen acceptor; the general equation is:

$$2H_2O + 2A \xrightarrow{h\nu} 2H_2A + O_2$$

This now well-known reaction has been shown both with "whole" chloroplasts and chloroplast fragments and with quinone, ferric salts, and such dyestuffs as dichlorophenolindophenol as hydrogen acceptor. Numerous other compounds have been added to this list including Janus green, cytochrome *c*, coenzymes 1 and 2, and flavin derivatives. As reported some years ago Davenport, Hill, and Whatley (1) have prepared from leaves a protein fraction which acts as a natural hydrogen acceptor catalyzing the reduction of methemoglobin by illuminated chloroplasts. Activity has now been shown to be confined to a component in the protein fraction which runs electrophoretically as a single band. This substance also catalyzes the reduction of cytochrome *c*. In the latter case whole chloroplasts from chard were used which had little cytochrome *c* oxidase activity (2). Both compounds, methemoglobin and cytochrome *c*, whose reduction is accelerated by the factor are heme compounds.

Whole *Chlorella* cells give in presence of ferricyanide a reaction apparently similar to the chloroplast reaction, but here, as shown by Warburg and Krippahl (3) and others previously, carbon dioxide is necessary. With *Chlorella* and quinone this is not so and the system essentially shows the chloroplast reaction. There is no evidence to show that for the chloroplast reaction carbon dioxide is essential. Many workers have shown photolysis in absence of any incorporation of radioactive carbon dioxide (4,5). There remains only the pos-

sibility that carbon dioxide is necessary in catalytic amounts, and if so this must then be in a form not freely exchangeable with external CO_2; there is no evidence to support this.

Until recently the maximal rates of oxygen production obtainable with chloroplast preparations at high light intensities ($Q_{O_2}^{Chl}$) were considerably lower than the corresponding rates of photosynthesis. This may be due to the use of unsuitable reagents, e.g., quinone. A very active isolated chloroplast system seems to be that obtained by Hill and Davenport using chard chloroplasts, the heme factor from pea, and cytochrome *c*. $Q_{O_2}^{Chl}$ of the order of 4000 have been obtained at 20°C., well within the range of values for photosynthesis.

KINETICS OF THE HILL REACTION; THE RELATIONSHIP BETWEEN RATE AND LIGHT INTENSITY USING THE "HEME" FACTOR

The chloroplast reaction is inhibited by many substances which inhibit photosynthesis, e.g., hydroxylamine, *o*-phenanthroline, and phenylurethane. The inhibition is similar to that of photosynthesis, and these results indicate that there are reaction steps common to the two systems. Other substances which markedly inhibit photosynthesis inhibit the chloroplast reaction only after prolonged exposure, e.g., cyanide, or not at all, e.g., SH inhibitors such as iodoacetamide and *p*-chloromercuribenzoate. Hence the primary photochemical reaction of photolysis need not require free SH groups for activity. On the other hand, the reduction of cytochrome *c* (or methemoglobin) in the presence of the Hill and Davenport factor is inhibited by *p*-chloromercuribenzoate and the inhibition is reversible by addition of cysteine (2). This presumably then is at least a two-step reaction in which the second link depends on SH groups; possibly the factor is itself unable to act catalytically without free SH groups. The participation of SH groups in chloroplast photochemistry cannot then be excluded except in the simpler photolytic reactions, e.g., quinone reduction.

The relationship between rate of oxygen production and light intensity has been compared for the Hill reaction and *Chlorella* photosynthesis. Clendenning and Ehrmantraut (6) compared oxygen production from *Chlorella* in presence and absence of quinone as a function of light intensity. They found the same initial slope (i.e., quantum efficiency) and maximal rate but a difference in rate for me-

dium light intensities. However, their observed maximal rates were low (in the case of photosynthesis by a factor of at least 5) and it is possible that maximal rates for the Hill reaction will be dependent on the oxidant used. Davenport, Hill and Whittingham (unpublished) have obtained light curves for pea chloroplasts in presence of the factor and methemoglobin, and it may be of interest to compare these

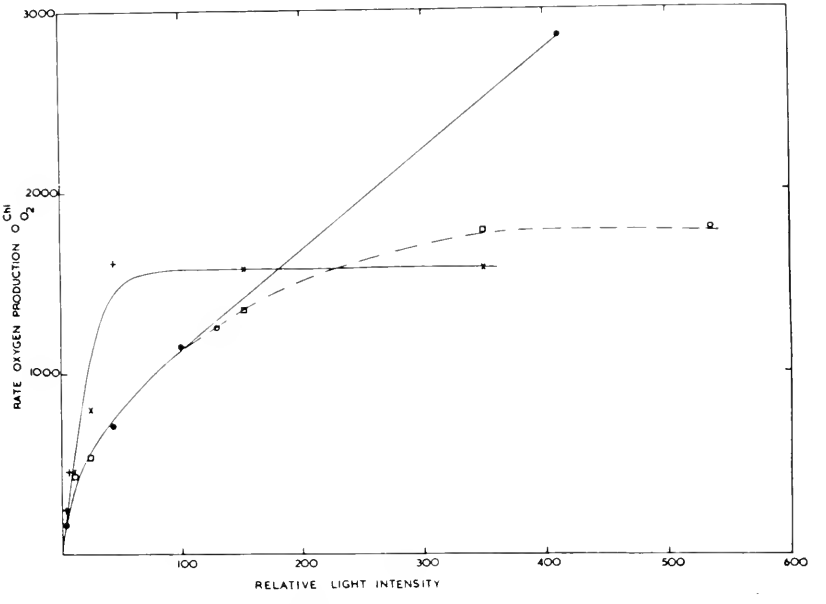

Fig. 1. The relationship between rate of oxygen production and light intensity for *Chlorella pyrenoidosa* (+, ×) and chloroplasts in presence of methemoglobin (● ○ □). Data for two independent chloroplast preparations are shown.

with the light curve for photosynthesis of *Chlorella* for the same absorption of light energy by chlorophyll.

The rate of oxygen production at different light intensities was compared for a suspension of *Chlorella pyrenoidosa* and chloroplasts isolated from *Pisum sativum*. Both rates were determined by the formation of oxyhemoglobin. With *Chlorella*, the cells were suspended in 0.033 *M* phosphate buffer (pH 6.7) with 3% carbon dioxide and the procedure was that described by Hill and Whittingham (7). The chloroplasts were suspended in 0.033 *M* phosphate buffer (pH

7.3) containing 6% glucose and KCl and the rate determined by reduction of methemoglobin as described by Davenport, Hill, and Whatley (1). The chlorophyll concentration of the two suspensions was adjusted to be the same, i.e., 1 mg. chlorophyll per 5 ml. suspension with an equivalent optical path of 1.3 cm. The hemoglobin concentration was 7×10^{-5} *M*. At the highest light intensity the rate of the chloroplast reaction was not increased by further addition of methemoglobin or "factor." The light source was a tungsten projection lamp; it was filtered of infrared radiation and adjusted by the use of neutral filters (Chance glasses ON32,ON31). Measurements were at 20°C.

The maximal rate of *Chlorella* used here was $Q_{O_2}^{chl}$ 1600; with the chloroplast preparation it varied between $Q_{O_2}^{chl}$ 1600 and 2900. It was found that despite differences in maximal rate and despite the fact that the two systems had the same rate at low light intensities, the chloroplast reaction always had a lower rate than photosynthesis at intermediate light intensities (Fig. 1).

Further information as to the nature of the photochemical reaction in photosynthesis and in the chloroplast reaction has come from studies with intermittent illumination. These were briefly discussed but are omitted here since they are referred to elsewhere in this book.

It might be concluded that there is a fundamental difference in the way in which water reacts with the photochemical system in the chloroplast reaction (at least as at present reconstituted) and in photosynthesis. For example, Franck has proposed that in the quinone reaction chlorophyll may act as hydrogen donor subsequently recovering the hydrogen by reaction with water, whereas in photosynthesis the chlorophyll is activated as a complex with the photosynthetic reactants.

REACTIONS OF CHLOROPLASTS OTHER THAN PHOTOLYSIS; THE ROLE OF CYTOCHROMES

Vishniac and Ochoa (8) showed that chloroplasts could in the light reduce DPN^+ and TPN^+; since that time it has been clear that in presence of a suitable enzyme system the reoxidation of this coenzyme by oxygen could be accompanied by phosphorylation. This type of oxidative phosphorylation, normally mediated by the cytochrome system, is dependent on the presence of oxygen or oxidized cytochrome, is inhibited by cyanide and uncoupled by DNP and methyl-

ene blue, and can be supplied with reduced coenzyme either in dark by addition of such acids as citric or malic or in light in presence of chloroplasts.

Two cytochromes, cytochrome *f* and cytochrome b_6 have been shown to be present in the green leaf (9) and appear to be peculiar to green tissue. Analogous cytochromes are present in the photosynthetic bacteria. Spectral changes (observed as difference spectra) indicate that in the bacteria and in the green plant these cytochromes are relatively more oxidized in light. (See contributions of Duysens, Chance, and others.) However reduced cytochrome *f* is not oxidized by oxygen in presence of chloroplast preparations; dismissing for the moment the problem of accessibility it may be that the plant has no corresponding oxidase and that oxidation in light is to be attributed to a photochemical product.

Recently light-induced phosphorylation in plastid preparations has been observed which appears to differ from oxidative phosphorylation in at least some respects. Frenkel showed that illumination of *Rhodospirillum* fragments in absence of oxygen resulted in phosphorylation of added ADP; further this reaction was not inhibited by DNP (10^{-4} *M*) or by iodoacetamide (10^{-3} *M*) but was inhibited rather weakly by cyanide (10^{-3} *M*). Whatley, Allen, and Arnon similarly found with "whole" chloroplast preparations from chard a light-induced phosphorylation which was inhibited by oxygen; it was stimulated by addition of vitamin K, ascorbic acid, FMN, and Mg^{++} but not by addition of coenzyme. Furthermore addition of Krebs cycle acid substrates in dark did not result in phosphorylation. Arnon was thus led to describe this reaction as "photophosphorylation." He was able to prepare from the same leaves a particulate fraction of smaller average size which did not show "photophosphorylation" in light but was able to phosphorylate in dark when supplied with suitable acids. This dark phosphorylation was dependent on oxygen and inhibited by the usual inhibitors for oxidative phosphorylation. Ohmura in attempting to repeat the work of Arnon *et al.* at first (10) obtained preparations of chloroplast fragments which gave oxidative phosphorylation in the dark when supplied with malic or citric acids but did not photophosphorylate. In later work he isolated the particles in presence of ascorbic acid (11). He then obtained a preparation of larger particles ("whole" chloroplasts) which showed "photophosphorylation" and no oxidative phosphorylation and another of

smaller particles which in light showed "photophosphorylation" and in dark oxidative phosphorylation. He suggests that since the same particles now exhibit both types of phosphorylation it is probable that "photophosphorylation" is simply the addition of photolysis and oxidative phosphorylation. The lack of dark oxidative phosphorylation by "whole" chloroplasts might be attributed to inaccessibility of the reaction system (compare the absence of cytochrome oxidase activity); the nonrequirement for oxygen of photophosphorylation suggests that the oxidized radical resulting from photolysis ("OH") can terminate the oxidative chain resulting in phosphorylation in absence of oxygen. The "OH" radical may react directly with a cytochrome.

THE CHLOROPLAST REACTION *IN VIVO*

Two types of mechanism have been suggested for the chloroplast reaction. The first is that electrons are transferred directly to a substance with an oxidation-reduction potential at least as negative as coenzyme. Calvin and colleagues have suggested that thioctic acid might be the primary electron acceptor (free energy per electron transfer 35 kcal.). The second type of mechanism is that a number of electrons are transferred as the result of absorption of a single quantum to a reagent of a potential relatively near that of oxygen. Thus four electrons could be transferred to cytochrome *f* (free energy per electron transfer 10 kcal.) with the energy from a single quantum. Subsequent dark reactions are required to produce a reducing agent by oxidative-reductive coupling until a potential near that of coenzyme is attained. One such chain was proposed by Davenport, Hill, and Whatley (1a) and Hill (9) to include cytochrome *f*, cytochrome b_6, and coenzyme. Wessels (12) has made the alternative suggestion that one quantum may transfer two electrons to vitamin K (free energy per electron transfer 19.5 kcal.) in the first stage.

It has been argued from the fact that the difference spectra for photosynthetic organisms indicate oxidation of at least some cytochromes in light that the first type of mechanism is more probable.

Discussion

Lucile Smith: I was wondering if you have been able to observe any oxidation-reduction effects of cytochrome *f* on illumination.

Whittingham: None at all. Cytochrome *f* after extraction is in the reduced form; when added to chloroplasts no oxidation takes place.

Clendenning: I would like to point out that in Dr. Thomas E. Brown's Ph.D. thesis, the temperature relations of photosynthesis and of the Hill reaction in the same cells are reported. This work was presented at the Paris meeting in July 1954 which many in this audience attended. Very much higher rates of the Hill reaction than of photosynthesis were observed at low temperatures, when using either whole *Chlorella* or *Nostoc* cells. Such comparisons should be with the Hill reaction in leaf chloroplasts. At 0°C., the Hill reaction rate in *Chlorella* was from 10 to 15 times higher than the full rate of photosynthesis, in aliquots of the same cell suspension, determined either by the two-vessel method or with number 9 buffer. On raising the temperature to 10°C., the Hill reaction rate was about four times higher. At 15°C., the Hill reaction rate was still above that of photosynthesis. At 20°C., the Hill reaction rate may be either above or below that of photosynthesis depending upon the duration of the experiments.

Rabinowitch: I think there is no doubt that, in strong light, the Hill reaction with quinone follows the laws of enzymatic reactions. From the experiments of Clendenning, it seems that there are two different temperature-dependent enzymatic reactions involved in photosynthesis, and that only one of them is left over in Hill's reaction. Franck has always maintained that, at lower temperatures—say 0 to 10°C.—the reaction that determines the temperature dependence of photosynthesis is different from the one which causes this dependence at higher temperatures—say 15° to 30°C.—and that the former reaction has something to do with the fixation of carbon dioxide.

At low temperature, the Hill reaction, which does not involve carbon dioxide, can therefore proceed much faster than photosynthesis; while at higher temperatures, a common reaction, having to do with the evolution of oxygen, limits both to the same maximum speed.

It is dangerous to draw conclusions from a comparison of the rates of the Hill reaction in chloroplasts from one plant with that of photosynthesis in another one. I don't see why cells of the same species as those from which the chloroplasts were prepared should not be used for comparison. I believe that, in a good chloroplast preparation, the maximal rate of oxygen evolution in bright light per unit chlorophyll amount will be found to be not slower than that of photosynthetic oxygen production—not necessarily in *Chlorella*, but in the same species from which the chloroplasts had been prepared.

Whittingham: That is merely a question of opinion in the absence of experimental data. I think if you have a suitable oxidant and a suitable system you may get the Hill reaction much faster on a chlorophyll basis than you do get photosynthesis.

Rabinowitch: I think the comparison should be with the same type of cells and not with *Chlorella*.

Whittingham: There is not a good Hill reagent that we can use with *Chlorella*.

Granick: With respect to the light curves for chloroplast and *Chlorella* how do you compare them?

Whittingham: Equal light absorption by chlorophyll.

Strehler: Did you say the quantum yield was the same in the two cases in the beginning?

Whittingham: Yes, as you extrapolate to zero light intensity we find that you always get the same rate. This is measuring the oxygen production under identical physical conditions.

Gaffron: I would like to put this question to Dr. Livingston and to Dr. Rabinowitch: Would it not be possible for the quinone reaction to occur in the singlet state of chlorophyll? After all, quinone does quench fluorescence! In this case, its reduction could occur with a better quantum yield than has been found so far. The temperature effect also indicates it to be nearer to the primary photochemical process and not so much subject to enzymatic limitations as is photosynthesis.

Livingston: If the quinone reaction involves the primary excited or fluorescent state, it probably could not be a diffusional reaction. The quinone would have to be associated with the chlorophyll or aggregated chlorophyll in the dark, as a thermally stable complex, or otherwise there would not be time enough for an efficient reaction to occur at these low concentrations of quinone.

Lumry: As far as higher plant chloroplast fragments are concerned, there are two distinct regions of the Hill reaction with apparently quite different mechanisms. These are controlled by oxidant concentration. At low concentrations there is a region which is completely independent of oxidant, and there is no quenching of fluorescence. But at the higher region, where a transition from one Hill reaction to another occurs, you begin to get quenching. The quenching goes along very smoothly parallel with the change from one Hill reaction to the other. So it may very well be that what Kamen suggested happens at the higher region though not at the lower.

Arnon: I would first like to make a point of some interest in connection with the role of chloroplasts in photosynthesis. Although I cannot recall at the moment the details of Hill's 1939 paper, in the 1950 paper, presented at Sheffield and published in the *Symposium of the Society for Experimental Biology*, Hill definitely envisaged chloroplasts as participating in the generation of some energy-rich compound, subsequently oxidized by molecular oxygen. Hill's idea was that in order to bridge the energy gap Kamen was talking about—that is, to compensate for the fact that no Hill reagent is a sufficiently strong reductant to accomplish the reduction of CO_2—a reoxidation of part of the primary reductant with molecular oxygen is necessary. His scheme was essentially analogous to that proposed by Warburg.

I wonder if Whittingham has a comment on that.

Whittingham: That is correct; but I think there is no evidence which would compel Hill to say that you could not replace oxygen in the reoxidation process, say, by a ferric complex or some other intermediate oxidant. As I see it, he used the simplest possibility—that of molecular oxygen as oxidant; but there was no compelling need for this assumption.

Arnon: My recollection is that he thought of this reoxidation as a respiratory process.

Whittingham: What he had in mind was some kind of oxidative reaction, not necessarily autooxidation.

Arnon: I wanted to find out whether Hill would object to the idea of the absence of respiration in chloroplasts—which we will suggest later—or whether respiration

is an essential element of his scheme. Kamen referred to Hill's early papers, while I am referring to his later work.

Whittingham: I don't think he considered oxygen as essential.

Arnon: This is a good point to have cleared up. The second question is this: It is rather important, insofar as photosynthesis by chloroplasts is concerned, to make sure where the cytochromes are localized in the green leaf. Have you any comment on that? Is there any evidence which would compel one to the conclusion that they are localized in the chloroplasts?

Whittingham: If you ask me for quantitative data, I have none. But I am quite certain that they are located, at least in part, in the chloroplasts.

Arnon: Are the cytochromes obtained from the chloroplasts after separating the latter from the cell?

Whittingham: Yes.

Chance: I want to make a short comment on Dr. Kamen's stimulating speculation concerning direct excitation of cytochrome in *Rhodospirillum rubrum*. It seems that the spectra of the forms involved in photosynthesis are very nearly identical with those involved in respiration. Therefore, if an excited state does exist, it seems to have no characteristic absorption spectrum—because the cytochrome molecules affected by light have the same bands as those affected by oxygen. Perhaps, excitation does not cause a measurable band shift: in the respiratory oxidative phosphorylation system, some of the enzymes probably exist in "high-energy" forms (at least, they can form high-energy phosphate bonds); yet, in this case, we do not detect any unusual spectral bands.

Kamen: This work emphasizes the need for reexamination of the spectra of the purified pigments. Up to now, I believe, nobody has ever had such pure forms to play with. In the particular case of bacterial cytochromes, the region where you would expect significant absorption bands would be in the near infrared. It is a matter of some difficulty to get cytochrome preparations so pure that you have the right to believe, when you measure the absorption spectrum, that a certain characteristic band can be assigned to this compound. In the case of cytochrome *f*, it has never been obtained in pure enough form so you could look for the band at 750 mμ, which one would expect to exist.

Arnon: I would like to offer a comment on Ohmura's experiments discussed by Dr. Whittingham. This also pertains to the question raised by Dr. Vishniac the other day. I think it is a simple yet important matter which greatly complicates discussions of chloroplasts and oxidative phosphorylation. Chloroplasts and mitochondria are both present in green cells, chloroplasts being much larger in size. Any technique used so far for breaking leaves will inevitably break some chloroplasts. Differential centrifugation will separate two fractions from the leaf. At least two. One would consist of whole chloroplasts and the other of broken chloroplasts plus mitochondria. The latter fraction is often referred to as "chloroplast fragments." This combined fraction is thus a mixture of broken chloroplasts and mitochondria. From this mixture the two kinds of particles are no longer separable on the basis of size.

I will now state the point which is documented elsewhere (*Biochimica et Biophysica Acta* (13)). If the discussion allows enough time we may bring the evidence in later. We find that photosynthetic phosphorylation, independent of

oxygen, is inherently a property only of whole chloroplasts. This is what Ohmura finds in his latest paper cited by Dr. Whittingham.

Brown: Broken fragments of chloroplasts contaminated with mitochondria could be doing phosphorylation.

Arnon: Yes, I am coming to that.

The mitochondria do only oxidative phosphorylation. But a "chloroplast fragments" preparation which contains both broken chloroplasts and some mitochondria can do both types of phosphorylation. The concept is very simple: there are two sites of phosphorylation in the cell, one strictly light-dependent, which is the property of the whole chloroplast, and the other independent of light but dependent on oxygen. The latter type, i.e., oxidative or respiratory chain phosphorylation localized in mitochondria keeps the cell going at night when the light is off.

To sum up, the green preparations known as "chloroplast fragments" are usually capable of the Hill reaction and also under certain conditions of some photosynthetic phosphorylation because they contain chloroplast material. These preparations may also be capable of oxidative phosphorylation because they contain mitochondria.

But the significant point, which is also reported in his last paper by Ohmura and explains his previous results, is the fact that whole chloroplasts do only photosynthetic phosphorylation when they are uncontaminated by other particles.

How much of each type of phosphorylation will be found in a given preparation depends on the relative proportions and activities of these two different components, one derived from chloroplasts and the other consisting of mitochondria.

Whittingham: I just want to ask for a point of information. Suppose you take a preparation of whole chloroplasts and throw away all the small particles and then break the whole chloroplast preparation. Will you find that the fragments from the whole chloroplast preparation exhibit properties which were not exhibited by the whole? For example, one might assay cytochrome *c* oxidase activity; have you done this experiment?

Arnon: Broken chloroplasts which have been prepared not from a mixed fraction but from a pure preparation of whole chloroplasts show only photosynthetic phosphorylation. However, we have prepared from the same spinach leaves three types of particles: whole chloroplasts, an intermediate fraction containing broken chloroplasts and mitochondria, and a third fraction containing all particles put together. We got oxidative phosphorylation or photosynthetic phosphorylation or both depending on how much of each component was present in the preparation.

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Natural Inhibitors of the Hill Reaction

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Although healthy green leaves invariably have high photosynthetic capacities, chloroplasts freshly isolated from them often have low or negligible capacities for the Hill reaction (1, 2). Hill (3) and McClendon (4) attributed this phenomenon to physical factors (plastid rupture, unfavorable osmotic conditions), but these do not account for the following observations: Chloroplasts from many species are consistently inactive when isolated intact in ice-cold sugar solution (1); chloroplasts having high Hill reaction capacities in the unbroken state retain this ability when finely disintegrated (5–7); the osmotic environment of leaf chloroplasts *in vivo* is far from constant (8, 9), and their Hill reaction activity *in vitro* is insensitive to osmotic pressure changes (10).

The first evidence of natural inhibitors was obtained by Kumm and French (1), who noted that inactive chloroplasts often were associated with tannins or related compounds which darkened Hill's solution. Clendenning and Gorham (2) demonstrated the presence of heat-stable, water-soluble inhibitors in inactive chloroplast sources, whose action was irreversible. Apart from this incidental information, little was known concerning natural inhibitors of the Hill reaction.

The grana of blue-green algae and the plastids of red algae retain Hill reaction activity only when isolated and tested in concentrated solutions of polymers such as dextrin (11) or polyethylene glycols (4). Phycocyanin and phycoerythrin otherwise are dissolved, with an accompanying loss of photochemical activity (11). McClendon (4) noted instances in which the capacity for water photolysis in green plant chloroplasts was improved by using 30% w/v Carbowax 4000 instead of 0.5*M* sucrose in their isolation, but this result was not obtained consistently. No stabilizing action of polyethylene glycols

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was observed by Bishop, Lumry, and Spikes (12) upon chloroplasts stored at low temperatures.

The studies summarized below involved several hundred experiments whose main purposes were the identification of natural inhibitors and the elucidation of the action of Carbowax upon chloroplast preparations. Chloroplasts were isolated and washed at 0 ± 1°C., and were maintained at 0°C. until tested manometrically with quinone at 10°C. (within 2 hours). The activity measurements were made on dilute chloroplast suspensions subjected to saturating orange-red light. Tannin analyses were made on aliquots of the supernate obtained in the first centrifuging of heat-coagulated chloroplast suspensions, tannin being calculated from the $KMnO_4$ titer before and after its complete removal with collagen. Chlorophyll was determined according to MacKinney (13). Leaf moisture was determined by drying at 100°C. Acidity of the leaf sap was assessed by grinding the leaves thoroughly with sand in two parts of distilled water and measuring the pH.

SAPONINS

Positive hemolysis tests for saponins were shown by over half of the leaf extracts examined in an extensive survey conducted by the Eastern Regional Research Laboratory, Philadelphia (14). Triterpenoid saponins were more commonly present than the steroidal type. Both of these classes include numerous molecular species. Our studies have indicated that the reversible inhibiting action of concentrated alfalfa leaf sap upon the Hill reaction is caused by its triterpenoid saponins. The steroidal saponin (sarsasaponin) of *Yucca* leaves was without effect at concentrations up to 1%. Inhibition by saponins in our experience has always been reversed by washing. In common with detergents, their inhibiting action resembles that of urethanes. Surface-active compounds presumably penetrate the lamellae at the protein-lipid interphases, where they become oriented along with the similarly polar chlorophylls and phospholipids. Transport of absorbed light energy may then be blocked as a result of their incorporation in the chlorophyll films (15).

VACUOLAR ACIDITY

When "active" chloroplasts are briefly immersed in leaf sap having a pH below 4.0 (or in dilute mineral and organic acid solutions of the

same pH), Hill reaction activity is lost irreversibly. This effect is eliminated, in the absence of other inhibitors, when the foreign leaf sap (e.g., *Oxalis*) is neutralized beforehand. Acid inactivation occurs so rapidly that chloroplasts isolated from acidic leaves possess no Hill reaction activity when the grinding fluid is strongly buffered with phosphate at neutrality—the plastids are subjected to the vacuolar sap during the homogenization which precedes release of the cell contents. Acid inactivation probably could be eliminated by soaking the leaves in dilute ammonia prior to maceration (16). This common type of chloroplast inactivation is unaffected by the use of concentrated Carbowax solutions.

Extensive information on the fluctuating acidities of leaf sap has been summarized by Small (17). The expressed sap of *Oxalis*, *Begonia*, and *Opuntia* leaves may have pH values as low as 1.4 when the leaves are collected early in the morning; by late afternoon the pH may rise to 5.5 in the same leaves. The sap of conifer needles (17) and of *Gingko* leaves usually has a pH below 4.0. Eight families of "acid" seed plants are listed by Small (17). The acidity gradient within the pineapple leaf observed by Sideris and Young (18) in itself would result in the inactivation of chloroplasts isolated from the apex (pH 3.3) but not from the base (pH 5.5) of the same leaf.

TANNINS

Tannins are common constituents of leaves (9) as well as of multicellular marine (19) and fresh-water algae (20). There are many different kinds of tannins, and the tannin from any one plant is often a mixture of polyphenolic compounds. Thus twenty-seven different polyphenols were obtained by countercurrent fractionation of *Acacia* extract (21). Identification of tannin as a chloroplast inactivator is readily accomplished with collagen: neutral aqueous extracts of certain leaves (e.g., *Rhus typhina*) which abolish the Hill reaction irreversibly in "active" chloroplasts lose this effect when the tannins are removed completely by selective adsorption on collagen.

Some evidence has been obtained of the variable effectiveness of different tannins as chloroplast inhibitors, and of varying contents of tannin within the same as well as related species. The leaves of mature honey locust trees (*Gleditsia*) regularly give positive histochemical tests for tannin; during chloroplast isolation, *Gleditsia* tannin remains insoluble and the chloroplasts possess high activity *in vitro*.

The leaves of small black locust seedlings (*Robinia*) contain negligible soluble tannin, and their chloroplasts possess high activity *in vitro;* the leaves of mature black locust trees contain tannin which is extracted with the chloroplasts, which show low or negligible capacities for the Hill reaction when isolated in neutral 0.5*M* sucrose or 0.05*M* phosphate. Tea leaf tannins are modified and degraded during tea manufacture (22); extracts of commercial tea often are ineffective as chloroplast inhibitors (2). When we collected *Spirogyra* from its natural habitat, tannin was regularly present; when samples were held for about one week in a laboratory aquarium provided with artificial light, the tannin disappeared. Every sample of red clover leaves (*T. pratense*) which we examined was rich in soluble tannins, and their chloroplasts were consistently devoid of Hill reaction activity *in vitro;* samples of white clover leaves (*T. repens*) collected simultaneously contained only traces of soluble tannin, and their chloroplasts were consistently active *in vitro.*

Chloroplast inactivation by leaf tannins probably has the same basis as the vegetable tanning of collagen, in which water of hydration is replaced by tannin (23). Although precise information is lacking, it is apparent that the action of tannins on different enzymatic systems differs greatly. So far as is known, the synthesis and degradation of tannins is accomplished enzymatically—tannase and polyphenol oxidase are two plant enzymes which must be quite insensitive to tannin. Tannins usually are abundant in tobacco leaves, whose chloroplasts possess only feeble activity *in vitro* (2); the fact that active polyphenol oxidase, cytochrome oxidase, and catalase preparations have been obtained from tobacco leaves (24) suggests that these enzymes are less sensitive to tannins than chloroplasts. Phosphatase (25), amylase (25), hyaluronidase (26), as well as phages (27) and influenza virus (28), on the other hand, are inactivated by low tannin concentrations.

The intracellular location of tannins was studied histochemically to learn why chloroplasts are photochemically active before but not after cell rupture in their presence. The palisade parenchyma of hard maple and sumac leaves contain tannin in their cell walls as well as in the cell sap. Tannin granules also were detected in maple leaf cytoplasm. Special cells (idioblasts) which were completely filled with tannin were also observed in these and other species. Tannin was concentrated in the end walls of the examined *Spirogyra* filaments.

Detectable amounts of tannin were found in the upper epidermal cells of *Phytolacca* leaves, which are excellent chloroplast sources. Spinach leaves were consistently free of tannins and the pH of their leaf sap was always above 6.0. The fact that tannin occurs within specialized photosynthetic cells (e.g., sumac, maple) is believed to establish that chloroplast inactivation by tannin as well as acids can occur by the homogenization of individual cell contents. Tannin is also contributed by nonphotosynthetic cells, and tannin which is located in the cell walls is brought into solution during chloroplast isolation. Here the temperature at which the chloroplasts are isolated is important—under otherwise standard conditions, the amount of tannin extracted with the chloroplasts usually increases when the maceration temperature is raised.

Having shown that the inactivation of chloroplasts which occurs during their extraction from many species is caused by tannins and acids, we attempted to devise a procedure which would yield active chloroplasts from acidic leaves of high tannin content. Sumac leaflets were used in these studies because of their acidity (pH 4.0 to 4.2) and exceptionally high tannin content. All of the extraction methods which we tested failed to yield sumac chloroplasts with measurable activity when the leaflets were macerated at 0°C. (large volumes of grinding fluid with and without Carbowax and sucrose, buffered at different pH values up to 9.0, and containing tannin adsorbents in excess (collagen, egg albumen); vacuum infiltration of the intact leaflets with buffered egg albumen solutions). Photochemically active sumac chloroplasts were eventually obtained by a very laborious method, the Hill reaction proceeding at a low but steady rate for 1 hour at 10°C. To obtain these, 5-g. intact leaflets were cooled to −70°C. in a medium consisting of 35 ml. fresh egg white diluted to 100 ml. with 0.2 *M* phosphate, pH 7.0, containing 0.2 *M* sucrose, 0.1 *M* Carbowax 4000, and 0.01 *M* KCl. The resulting cake was chipped and then ground to a fine powder at *ca.* −70°C. in the presence of excess dry ice. The melted brei was processed in the usual way, employing large volumes of the foregoing medium as wash liquid. The fact that photochemically active sumac chloroplasts were obtained when the leaflets were ground at −70°C. but not at 0°C. in the same maceration medium strongly indicates that chloroplast inactivation in this leaf is an intracellular phenomenon.

CARBOWAX-TANNIN INTERACTION

The first indication of Carbowax-tannin interaction was obtained when the collagen method for tannins was applied to leaf extracts containing 30% Carbowax 4000. Tannins are readily removed by collagen from leaf extracts prepared with 0.5 *M* sucrose or 0.05 *M* phosphate, but they are not removed completely from Carbowax extracts even when the amount of collagen is trebled. Further experiments showed that Carbowax also interferes in the adsorption of tannin by chloroplasts. The foregoing phenomena apparently are caused by the affinity of Carbowax for tannin in solution: leaf tannins are removed from simple aqueous solutions by liquid-liquid extraction with ethyl acetate, but under otherwise similar conditions they are not extracted by ethyl acetate from 30% Carbowax 4000 solutions. The affinity of Carbowax for tannins in solution probably was also responsible for its frequently observed inhibition of the enzymatic browning reaction in leaf macerates (which results from the action of polyphenol oxidase upon "tannins" (29)).

Because of the above effects of Carbowax upon tannins, one might expect to obtain active chloroplasts from certain species with Carbowax, though the same species yield essentially inactive chloroplasts when ground in sucrose solutions, etc. Several examples of this favorable effect of Carbowax were encountered (walnut, red oak, hard maple, black locust, and geranium leaves). Examples also were encountered, however, of leaves which contained sufficient soluble tannin for chloroplast inactivation when 30% Carbowax 4000 was employed as grinding medium at 0°C. (sumac, red clover, ragweed leaves).

Chloroplasts which exhibit negligible Hill reaction activity after mild washing rarely show improvement upon exhaustive washing, but this is a characteristic of red elm chloroplast suspensions. When these chloroplasts were isolated at 0°C. in accordance with McClendon (4), they exhibited oxygen absorption in light when neutral 30% Carbowax 4000, 0.5 *M* sucrose, and 0.05 *M* phosphate were employed as grinding and dispersion media. The supernates did not inhibit active chloroplasts, and the soluble tannin content was no higher than in several "active" species; the leaf sap also was approximately neutral. After four washes with 30% Carbowax 4000, the red elm chloroplasts which previously were "inactive" now exhibited

TABLE I. Hill Reaction Activity of Chloroplasts Freshly Isolated, Washed, and Resuspended at 0°C. in 0.05 *M* Phosphate-Buffered 30% Carbowax 4000, 0.5 *M* Sucrose, and Buffer at pH 7.0. Tested with 1 mg. Quinone at 10°C., in Orange-Red Light (70,000 ergs/(cm.²/sec.) at Vessel). Activity Calculated from O_2 Evolution during First 20 Minutes in Light

Chloroplast source	% solids	Cell sap, pH	Tannin, mg./ml. supernate	Chlorophyll, mg./vessel	Mm.³ O_2/hr./mg. chlorophyll at 10°C.		
					Carbowax	Sucrose	Phosphate
Acer saccharum (sugar maple)	43	4.2	4.6	0.2	350	105[a]	65[a]
Robinia Pseudo-Acacia (black locust)	47	6.6	2.7	0.2	250	60[a]	45[a]
Cercis canadensis (red bud)	36	4.6	0.7	0.1	615	310	175
Gleditsia tricanthos (honey locust)	54	5.3	0.4	0.2	985	535	510
Gymnocladus dioica (Kentucky coffee tree)	37	5.8	0.3	0.3	710	385	385
Ailanthus altissima (tree of heaven)	27	5.7	0.3	0.2	395	260	310
Medicago sativa (alfalfa)	31	6.3	0.3	0.2	815	625	550
Trifolium repens (white clover)	25	6.2	0.3	0.2	545	395	400
Elodea canadensis (water weed)	9	6.8	0.2	0.2	600	450	310
Lemna minor (duckweed)	6	6.9	0.2	0.1	540	395	350
Phytolacca americana (pokeweed)	13	6.2	0.2	0.2	960	665	785
Spinacea oleracea (spinach)		6.8	0	0.1	730	350	290

[a] Inactive after 15 minutes.

high Hill reaction rates ($Q_{O_2}^{Chl}$ = 1000 at 10°C.). Chloroplasts of this species are characterized by high polyphenol oxidase activity, and the leaf macerates are extremely viscous. The polysaccharide causing the viscosity, however, is not in itself responsible for the initially low activity: the polysaccharide was isolated from "blanched" leaves as well as from the inner bark according to Gill, Hirst, and Jones (30); upon providing similar viscosities with the polysaccharide from these two sources, the Hill reaction rates of "active" chloroplasts were not inhibited by more than 30%. The remarkable improvement of red elm chloroplasts by exhaustive washing cannot be explained in terms of saponins, acids, or tannins, and so should be ascribed to a fourth type of interference.

CHLOROPLAST STABILIZATION BY CARBOWAX*

When leaves are ground in 30% Carbowax 4000, the cytoplasm is precipitated on the chloroplasts. (The supernate from the first centrifugation is devoid of heat-coagulable protein.) This "salting out" phenomenon is regularly associated with an increased stability of the chloroplasts' capacity for water photolysis. When the chloroplasts are freed of cytoplasm by isolation in 0.5 *M* sucrose, they do not exhibit enhanced stability when subsequently suspended in 30% Carbowax 4000. The preservative effect of Carbowax which is exerted via the cytoplasm is magnified by prolonged storage and by the use of relatively high temperatures in isolating and testing the chloroplasts. It is also exhibited, however, in freshly isolated chloroplasts (isolated at 0°C., tested at 10°C.), provided limiting numbers of chloroplasts are used in the activity measurements. Examples were encountered of chloroplasts which showed no higher initial activity when isolated in 30% Carbowax 4000 vs. 0.5 *M* sucrose, just as McClendon observed (4). However, with the use of successively smaller aliquots of such chloroplast suspensions (e.g., *Ailanthus altissima*) the observed capacity for the Hill reaction regularly became higher in the Carbowax than in the sucrose preparations. The different results obtained with Carbowax vs. sucrose by McClendon at two stations (4) may have been caused by the provision of chloroplast-limiting conditions at one station and not the other.

* Further information on this subject is reported by the authors in *Physiologia Plantarum, 9:* 519–532, 1956.

OSMOTIC PRESSURE

The leaf sap of land plants is characterized by widely different and fluctuating osmotic pressures (8,9). The osmotic pressures to which chloroplasts are subjected within a single leaf are far from constant. Gradients exist between different leaf tissues, the osmotic pressure regularly being higher in palisade than in spongy parenchyma (8). The osmotic pressure of the leaf sap undergoes diurnal and seasonal changes, and also varies with the leaf position, growth environment, and species (8,9). According to McClendon (4), it has been known for almost seventy years that the swelling of isolated chloroplasts can be prevented by sucrose solutions. A maceration medium of constant composition matches the fluctuating natural environment of leaf chloroplasts only sporadically, even in leaves of a single species. We have compared the photochemical activity of chloroplasts isolated in neutral 30% Carbowax 4000, 0.5 *M* sucrose, and 0.05 *M* phosphate, using "active" sources ranging from the "leathery" leaves of several trees to simple aquatic plants. The measured water contents ranged from 46% in *Gleditsia* to 94% in *Lemna* (Table I). The stabilizing effect of Carbowax was apparent in chloroplasts obtained from all these diverse sources, provided limiting amounts of chloroplasts were used in the activity measurements. The photochemical activities of chloroplasts isolated in 0.5 *M* sucrose and 0.05 *M* phosphate were approximately the same. The much higher activity of the maple and black locust chloroplasts in Carbowax vs. sucrose is attributed to Carbowax-tannin interaction. The proportionately smaller enhancement of the initial Hill reaction rate in chloroplasts from species of low soluble tannin content is attributed to the stabilizing action of Carbowax via the cytoplasm. *Elodea* is one of the several plants which have yielded active chloroplasts in one survey (4) and not another (2); this may be a result of varying concentrations of natural inhibitors. The *Elodea* samples used in the present study yielded active chloroplasts consistently, with evidence of the usual preservation effect of Carbowax. Several new sources of "active" chloroplasts (woody and herbaceous legumes) are reported incidentally in the accompanying table.

The natural inhibitors discussed above represent those which have been positively identified in a survey of a few dozen species. Many additional types might be disclosed by further study. The best plant sources of chloroplasts, mitochondria, and soluble enzymes are those which are consistently free of irreversible inhibitors.

Discussion

Blinks: You are inclined to rule out effects due to osmotic properties of the plastid?

Clendenning: In selecting species for this study, we included chloroplast sources of widely different osmotic pressures, ranging from aquatic plants to tree leaves containing over 50% solids. Over this extreme range of cellular osmotic pressures, the chloroplasts' capacity for water photolysis *in vitro* was consistently improved by the use of 30% Carbowax 4000.

Bishop: What about the relative stability of chloroplasts at room temperature in sucrose and Carbowax solutions?

Clendenning: In our experience, chloroplasts isolated and tested in 30% Carbowax 4000 at limiting chloroplast concentrations have shown higher photochemical activity than in 0.5 *M* sucrose, this effect of the Carbowax becoming stronger as the reaction temperature is raised. I think that the main way in which the Carbowax acts is by stabilizing the chloroplasts after isolation, and that it does so by surrounding the plastids with precipitated cytoplasm. It should be pointed out that 30% Carbowax 4000 precipitates egg albumin from aqueous solutions.

Granick: I was interested in why the chloroplast can't be preserved with sucrose, if this really is an osmotic effect. Don't you think it may also be an effect of the size of the molecules?

Blinks: Precisely that. Sucrose penetrates and the polyethylene glycol does not. A redistribution of the phycoerythrin occurs with red algal plastids suspended in sucrose solutions but not in concentrated polyethylene glycol solutions.

Arnon: In working with particles such as mitochondria or chloroplasts what criterion should we use for distinguishing between physiological and nonphysiological substances, excepting substances such as versene which are admittedly nonphysiological? Dr. Jacobs included magnesium in the nonphysiological ones. What is your criterion for saying this?

Jacobs: A good set of criteria is hard to find. For instance, in the intact system there is no magnesium requirement. Under certain conditions it will show up. Since magnesium is such a good physiological substance, it is assumed that it is an actual physiological requirement. But as far as these other things go—like, say, manganese—unfortunately, we have no criteria.

Brown: Operationally, there seems to be no distinction that one can make.

Arnon: That is the point.

Gaffron: Is sodium chloride not a case in point? A plant grown without chloride preserves its capacity to photosynthesize and yet it is known that the chloroplast does better if sodium chloride is added.

Arnon: Yes, I am very glad that we were cautious in stating that the nonessentiality of chloride for photosynthesis was based on the assumption that chloride is not essential for intact green cells. Recent evidence by my colleagues at Berkeley suggests that chloride may have to be added to the list of essential elements for higher plants. Thus the original proposal of Warburg that sodium chloride may be a cofactor in photosynthesis is not ruled out by our argument made at that time.

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Light-Dependent Reductions in a Cell-Free System*

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A few years ago I reported on a cell-free system obtained from spinach leaves which could be shown to require chlorophyll for a light-dependent reduction of TPN. To recapitulate briefly, green granules were obtained from macerated leaves and washed repeatedly by centrifugation. The particles were then extracted with acetone at −5°C., on occasions also with butanol, until the washings were colorless. An aqueous extract of the resultant greenish powder yielded a colorless or yellowish solution; 2.0 ml. of such an extract in phosphate buffer at pH 7.0, mixed with 0.05 ml. of an ethanolic extract of lyophilyzed chloroplasts and incubated anaerobically in the light, reduced TPN as measured by the reduction of oxidized glutathione via a TPN-dependent glutathione reductase. The upper part of Table I shows such an experiment.

More recently attempts have been made to fractionate the protein portion of this reaction mixture. The lower portion of Table I indi-

TABLE I. Reduction of Oxidized Glutathione

	Reduced glutathione formed (micromoles)
Complete reaction mixture[a]	1.3
No TPN	0.4
No GSSG reductase	0.4
No acetone powder extract, or heated extract, or egg albumen substituted	0
Ammonium sulfate fraction of acetone powder extract	
0–0.2	0.6
0.2–0.5	1.8
0.5–1.0	0.7

[a] The complete reaction mixture contains: acetone powder extract, 3.0 mg. protein; ethanolic extract, 0.1 mg. chlorophyll; oxidized glutathione, 10 μM; TPN, 0.1 μM; $MgCl_2$, 1.0 μM; glutathione reductase. Time, 20 minutes; temperature, 15°C.; illumination, incandescent light.

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cates that a major portion of the activity resided in a fraction precipitable at 0.2 to 0.5 saturation of ammonium sulfate. In a similar series of experiments with dandelion leaves most of the activity could be precipitated at 0.1 to 0.3 saturation of ammonium sulfate. The oxidized product formed in this reaction is unknown.

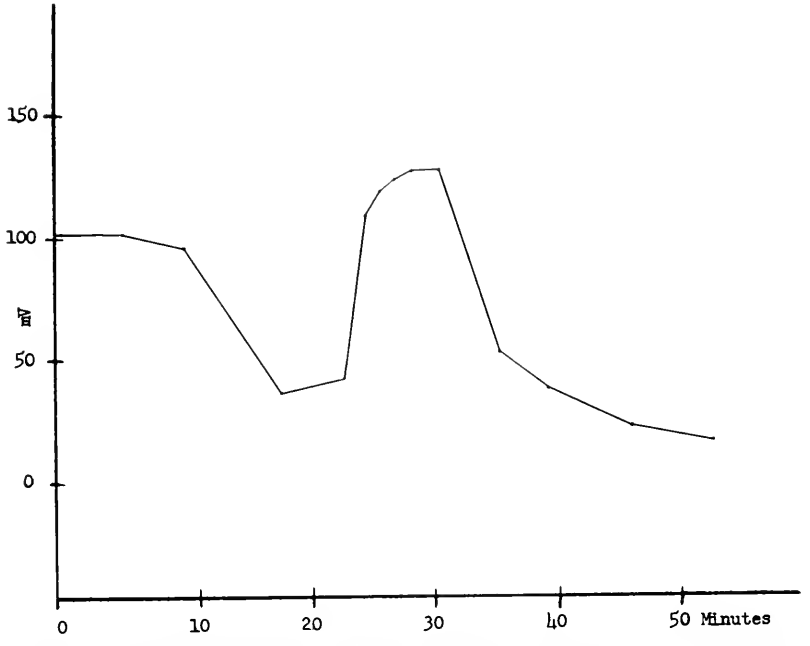

Fig. 1. Potential changes in the light. Reaction mixture: 1.0 ml. extract of chloroplast acetone powder in 0.05 *M* K-PO_4, pH 7.0, containing 1.0 mg. protein; *p*-quinone, 1 mg.; to which was added later ethanolic extract, 0.05 mg. chlorophyll. Temperature, 15°; illumination, incandescent spotlight; time in minutes 0, dark, flushed with N_2; 5, light turned on; 10, chlorophyll added; 18, light off; 23, N_2 replaced with O_2; 31, O_2 replaced with N_2 and light turned on.

The activity determined by the enzymatic assay appears to parallel reducing activity which can be determined potentiometrically (1). A reaction vessel containing a platinum-calomel electrode pair was used, in which 2 to 3 ml. of a reaction mixture could be stirred by a continuous stream of gas. With quinone the results shown in Fig. 1 were obtained before the platinum electrode was ruined. Since the

oxidized product formed in this reaction is not known, we cannot interpret the potential changes in stoichiometric terms.

Discussion

Frenkel: Did you see any evidence for oxygen evolution in this?

Vishniac: I didn't look for it.

Frenkel: You would not expect it?

Vishniac: Something must be oxidized to balance the reduction, but this is rather rough treatment for a *chloroplast* to expect oxygen evolution.

Frenkel: Has it worked with ferricyanide?

Vishniac: Originally I reported that there was an effect of ferricyanide, and I thought it participated stoichiometrically by being oxidized, but this is not so. In these experiments ferricyanide was added simply to have an electrode-active electron carrier.

Rabinowitch: These figures refer to a certain time; if you had waited longer would you have obtained more?

Vishniac: The actual time of these experiments is 20 minutes. I have not run any longer experiment, but in 10 minutes I get about 1 μM, that is, one-half of this.

Rosenberg: Does the alcohol extract of chlorophyll before reconstituting with the protein give the fluorescence that is expected of regular alcohol solutions?

Vishniac: I don't know.

Rosenberg: You can generally see this with your eye.

French: Does pure chlorophyll work too?

Vishniac: I have not tried that.

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Photosynthetic Carbon Dioxide Fixation by Broken Chloroplasts*,†

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Whole chloroplasts isolated from spinach leaves by grinding in 0.35 *M* sodium chloride or 0.5 *M* sucrose have previously been found to fix carbon dioxide when illuminated (1,2). The fixation of carbon dioxide was strictly light-dependent (1,2). The rate of carbon dioxide uptake was proportional to the amount of chloroplasts added, indicating that all the components necessary for the process were contained in the chloroplast (2).

Further investigation showed that illuminated whole chloroplasts carried out the complete process of photosynthesis. Fixation of carbon dioxide was found to be accompanied by the evolution of an approximately equivalent quantity of oxygen (2). Examination of the compounds formed from carbon dioxide showed that 30% to 40% of the CO_2 fixed was converted into starch (2). A study of the soluble compounds by means of paper chromatography and radioautography revealed a pattern similar in some respects to that found after assimilation of carbon by whole cells. The principal products identified in the chromatograms were mono- and diphosphates of hexoses and pentoses and dihydroxyacetone phosphate. Glycolic and malic acids, alanine, glycine, and free dihydroxyacetone were also found (2), as was free glucose.

Until very recently, photosynthesis by isolated chloroplasts was considered to be strictly dependent on the structure of the intact chloroplast. Disruption of this structure by treatment with water resulted in the complete loss of ability to fix CO_2 (3,4). However, it was found

* Abbreviations: P_l, whole chloroplasts; P_{lw}, water-treated chloroplasts; CE, chloroplast extract; ATP, adenosine triphosphate; DPN, TPN, di- and triphosphopyridine nucleotides; FDP, fructose-1,6-diphosphate.

† Aided by grants from the National Institutes of Health and the Office of Naval Research.

that a large proportion of the protein of the chloroplast was extracted by the water treatment. The ability of the broken chloroplasts to fix CO_2 was partially restored when the water extract of chloroplasts was added back to the residual particles, as shown in Table I. As with whole chloroplasts, CO_2 fixation by the broken chloroplast system was strictly dependent on illumination.

TABLE I. Effect of Chloroplast Extract (CE) on Photosynthetic Carbon Dioxide Fixation by Broken Chloroplasts (P_{lw}) (4)

Expt. No.	$C^{14}O_2$ fixed (c.p.m.)		
	P_{lw}	P_{lw} + CE	P_{lw} + CE dark fixation
54	2,700	108,300	14,700
56	1,950	149,400	7,200
65	1,900	276,800	24,400
67	950	137,300	—

The water extract of chloroplasts was found to contain a number of enzymes. Those so far identified are:

phosphorylase	menadione reductase
amylase	pentose phosphate isomerase
phosphoglucomutase	phosphoribulokinase
hexose phosphate isomerase	carboxylating enzyme (5)
aldolase	transketolase

TPN- and DPN-dependent triosephosphate dehydrogenases (6).

The addition to the broken chloroplasts of DPN, TPN, and ATP, singly or in combination, failed to restore their capacity for CO_2

TABLE II. Effect of Adenosine Triphosphate (ATP) and Di- and Triphosphopyridine Nucleotides (DPN and TPN) on the Fixation of $C^{14}O_2$ by Broken Chloroplasts Supplemented with a Water Extract of Chloroplasts (CE) (4)

Additions	$C^{14}O_2$ fixed (c.p.m.)		
	P_{lw}	P_{lw} + CE	P_{lw} + heated CE
None	950	137,300	19,750
ATP	1,300	204,950	35,400
DPN	2,250	347,700	20,250
TPN	1,600	450,250	19,450
ATP + DPN	2,000	411,950	35,550
ATP + TPN	2,800	659,350	29,900

TABLE III. Effect of Phosphorylated Compounds on Photosynthetic Carbon Dioxide Fixation by Broken Chloroplasts (10 μmoles CO_2 added)[a]

Additions	CO_2 fixed, μmoles
None	2.0
3-Phosphoglyceric acid	3.7
Ribose-5-phosphate	4.0
Glucose-1-phosphate	4.4
Glucose-6-phosphate	3.8
Fructose-6-phosphate	3.1
Fructose diphosphate	5.2

[a] 3 μmoles of each phosphorylated compound was added to the reaction mixture. Other experimental conditions as described in (4).

fixation in the absence of added chloroplast extract (CE), but increased photosynthetic CO_2 fixation in the presence of CE. Heating

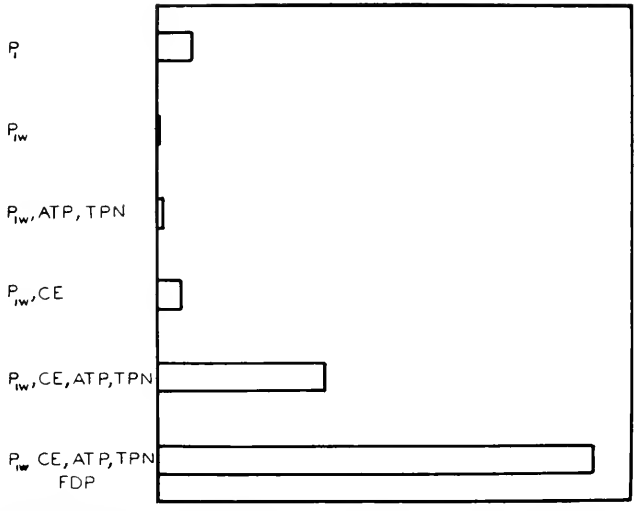

Fig. 1. Diagrammatic representation of the effects of various addenda on CO_2 fixation by broken chloroplasts. Experimental conditions as in (4).

the CE almost completely abolished its effect on CO_2 fixation by broken chloroplasts. The effects of added pyridine nucleotides and ATP, with and without CE, are shown in Table II.

A further marked increase in CO_2 fixation by the broken chloroplast system was obtained on adding small quantities of any one of a number

of phosphorylated sugars and related compounds, as shown in Table III. Figure 1 illustrates schematically the loss of photosynthetic activity of whole chloroplasts by treatment with water and its restoration by the various addenda. The level of photosynthetic CO_2 fixation by the reconstituted system was several-fold that originally obtained with whole chloroplasts.

Fig. 2. Chromatogram showing products of photosynthetic CO_2 fixation by broken chloroplasts. Compound directly above area labeled "monophosphates" has been identified as phosphoglyceric acid. Experimental procedures as given in (2) and (4).

These experiments with broken chloroplasts were carried out in the presence of ascorbate and manganous ions, which were previously found to stimulate carbon dioxide fixation by whole chloroplasts (2). The omission of Mn^{++} from the broken chloroplast system reduced CO_2 fixation to 20% to 30% of the usual value—a much more marked effect than that previously noted with whole chloroplasts.

The products of carbon dioxide reduction by the broken chloroplast system, shown in Fig. 2, resembled in most respects those obtained from whole chloroplasts. The proportion of insoluble material was smaller, but could be increased by the addition of soluble starch, suggesting a deficiency of primer for starch synthesis. Malic and aspartic acids have not been found. Phosphoglyceric acid, which was present only in traces among the compounds formed by whole chloroplasts, was found to be a more conspicuous product of the broken chloroplast system.

Discussion

Witt: What about the size of the broken chloroplast?

M. B. Allen: That is a little hard to answer. There is a progressive change. One starts off with a chloroplast that is smooth in outline, rather elliptical. The first thing that happens is that the smooth outline is blurred and the chloroplasts begin to look irregular. As they are allowed to stand in water the whole structure more or less falls apart and we get globs and bits.

Witt: How were the chloroplasts heated?

M. B. Allen: The chloroplasts were not heated. The chloroplast extract was heated at 100°C. for 90 seconds.

James Smith: Do you get starch?

M. B. Allen: We get some starch. For a reason that we don't fully understand, the quantity of starch formed by the broken chloroplast is far less than that formed by whole chloroplasts. We find that we can increase it a bit by adding a little soluble starch, but in no case does it rise beyond 30% or 40% of that obtained with whole chloroplasts.

Fuller: I would like to make one comment about whether grana contain carboxylating activity. Dr. Allen pointed out that there is no direct evidence for this in the green leaf. However, work has been done in our laboratory which indicates that, as far as carboxylating enzymes are concerned, the grana (if you want to call them grana) of *Rhodospirillum*, and of blue-green algae as well, prepared in the same way, do contain at least the carboxylase of photosynthesis. Other observations can be added to this. By bleaching the cells of *Euglena* with streptomycin and growing *Euglena* in the dark, at least the primary carboxylating enzyme is lost. There are cases in albino plants where the same loss of activity has been observed with the carboxylating enzyme and some additional enzymes concerned in photosynthesis.

Strehler: Dr. Allen, how does the maximum rate of CO_2 fixation obtainable with your chloroplasts compare to photosynthesis in the whole cell, e.g., on a chlorophyll basis?

M. B. Allen: This is a slightly difficult question to answer for leaves. We have this on our agenda to investigate.

Arnon: This question comes up very often and, as Dr. Allen has indicated, we are going to do the experiment, but not because we believe it is important at this

time. This is a strange admission to make, but I will tell you why we don't think it is important. Krebs has pointed out in his review in 1954 (*Chemical Pathways of Metabolism*, *1*, 109 (1954), D. M. Greenberg, ed., Academic Press, New York) that today, fifty years after respiration was first obtained outside the living cell, we can obtain only 10% of the total respiration of the yeast cell by putting all the known enzymes and coenzymes together; yet one of the great contemporary biochemical advances has been the understanding of the enzymatic apparatus of respiration by studying and trying to put together the isolated enzyme system.

Strehler: How does your rate compare with *Chlorella* on a chlorophyll basis?

Arnon: We have not made that computation. What we think is important is the fact that we get an appreciable and reproducible complete photosynthesis by isolated whole, and now also by broken, chloroplasts. CO_2 fixation by broken chloroplasts can be increased by 500% by adding certain substances. However, we definitely have on the program an experiment to compare the rate of photosynthesis by isolated chloroplasts with that of the same leaves from which the chloroplasts were prepared.

Strehler: The previous question leads to the following one: Assume that the ratio of CO_2 fixation in the chloroplast is essentially the same as the fraction of the whole cell volume the chloroplast itself occupies. If in your isolation procedure you form some membrane around the chloroplast—a coagulation membrane of some sort which traps these enzymes in association with the chloroplast (such as has been demonstrated for isolated liver nuclei)—then one need not necessarily assume that the chloroplast has all the necessary enzymes concentrated in it or that it is acting as a unique photosynthesizing unit. Rather it may merely be floating around in a cytoplasm which itself contains all the necessary enzymes.

Gibbs: As a biochemist I am trying to determine what this group has accomplished that is new. Dr. Arnon has stated on various occasions that his chloroplast preparations possess a pathway for the conversion of CO_2 to carbohydrate other than that postulated by Benson, Calvin, Horecker, *et al.*, for the intact cell. Your data do not show a new enzyme. They do not suggest a new compound. I do not see in the data a new pathway for a conversion of CO_2 to carbohydrate. What interests me most in these data is the elegant manner in which you have shown that the chloroplast apparently possesses a complete set of enzymes for the carbon cycle independent of those found in the cytoplasm for carrying out similar reactions. This localization may be a clue to the manner in which the plant separates carbon of photosynthesis from carbon of respiration.

Arnon: Let me say first that we are not defending anything we have done or its significance. Our first aim was to show where photosynthetic CO_2 fixation and ATP formation are localized in the cell. One might say that it is unimportant to have a Hill reaction or any other reaction by isolated chloroplasts. Why take a chloroplast out of a leaf when we know that the whole leaf can give off oxygen? Yet, as we have seen at this meeting and from the published research on photosynthesis, there is a great deal of interest in what isolated chloroplasts can do when taken out of cells.

Strehler: Do the isolated chloroplasts contain adsorbed cytoplasmic enzymes?

Arnon: Now then, may I proceed to Dr. Gibbs' point next? As far as my statement at the last year's (1954) Botanical Congress is concerned, I am afraid that

you inaccurately quoted what I said, and I wish to correct that inaccuracy now because I don't want to be misquoted again. What I said last year, and what I am saying now, is that we are keeping an open mind on the path of carbon in photosynthesis. That does not mean that we are accepting or rejecting the present concept. What we are saying is that, if the entire photosynthetic process is localized in chloroplasts, then we must find in them finally a pathway for carbon fixation that would be the same as in the whole cell. That we have not done yet. For that reason we do not accept at this time the Calvin-Horecker pathway because we don't as yet have the evidence that would permit us to say that this is the pathway of carbon fixation in the chloroplast.

As for the point of adsorption of enzymes raised by Dr. Strehler, I think there is a great deal of misunderstanding as to just what is being done in getting chloroplasts out of the cell. In the intact cells you have chloroplasts which are large particles in relation to mitochondria. We separate chloroplasts because of their large size by differential centrifugation and we throw away everything else. So any major contamination is removed initially. This is Point 1.

Point 2. We wash these isolated chloroplasts once.

Point 3. We add the remaining cytoplasmic constituents to the chloroplasts. On the hypothesis that the active constituent in CO_2 fixation and ATP formation is some small contaminant remaining on the chloroplasts after differential centrifugation and washing, we would expect an increase in activity when we add all the rest of the cytoplasm to the chloroplasts. However, no such effect is found.

Point 4. The fixation of the CO_2 is proportional, as you have seen on the slide shown by Dr. Allen, to the chlorophyll concentration in the chloroplasts.

Point 5. Directly in answer to Dr. Strehler's question—we can now take whole chloroplasts, break them, thus removing this hypothetical coagulation barrier which is supposed to trap extraneous enzymes, without losing any capacity for ATP formation by the remaining chloroplast fragments. Capacity for CO_2 fixation is restored to these broken chloroplasts by the addition of a water extract of whole chloroplasts.

We feel that these five points render it extremely unlikely that there is any significant enzymatic contribution from outside the chloroplast.

Kok: We must distinguish our attacks on the problem. I do feel that it is not a fair statement to say that Hill's publications have shown only that the chloroplast is something special. Hill has shown that the mechanism of photosynthesis, can be broken apart, that the photolysis of water, which generates the reducing power, can be fully separated from the process of carbon dioxide conversion.

Arnon: There is one simple point, Dr. Kok, that is very important. The cell is a very large system. We have all kinds of enzymes in there. If you can be sure that all the enzymes in photosynthesis are localized on one place in the cell, that at once gives you an enormous simplification of the problem because you can look for them in the chloroplast and you reduce the chance of confusing the respiratory cycle or other metabolic cycles with photosynthesis. That is why these problems of localization of cellular enzymes and their function are related.

The first point in our approach was to localize the site of complete photosynthesis in chloroplasts. What comes next is getting the enzymes out from the chloro-

plasts. Then we will not have to worry about contamination from enzymes that came from mitochondria.

Krall: I have made a comparison of Dr. Arnon's published rates of $C^{14}O_2$ uptake with the rate of photosynthetic $C^{14}O_2$ fixation by barley leaves in 2% CO_2. This may not be a fair comparison because the barley leaf is a much more active system than the spinach leaf. Using Arnon's highest value, the calculations show that barley fixes C^{14} at about 1000 times the rate of chloroplast preparations.

M. B. Allen: With the reconstituted system we have obtained far higher rates than that.

Arnon: I question the validity of Dr. Krall's comparison.

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General Concept of Photosynthesis by Isolated Chloroplasts*

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The old concept, once dominant in plant physiology, that the entire photosynthetic apparatus is localized in chloroplasts was discredited about twenty years ago when Hill showed that isolated chloroplasts could not fix CO_2 although they retained a capacity to evolve oxygen when illuminated in the presence of an artificial electron acceptor in accordance with equation 1.

$$\text{Light} + A + H_2O \longrightarrow H_2A + {}^1/_2O_2 \qquad (1)$$

in which A represents an electron or hydrogen acceptor other than carbon dioxide. The failure of isolated chloroplasts to fix CO_2 was later confirmed with isotopic carbon [see review, 4]. The old concept was never based on direct evidence for CO_2 fixation by chloroplasts but on the view that the oxygen of photosynthesis came from the splitting of CO_2; hence oxygen evolution by chloroplasts, first observed by Engelmann in the 1880's, was accepted as evidence of simultaneous CO_2 fixation.

In the light of modern knowledge equation 1 has, until recently, represented the only known photochemical activity of isolated chloroplasts, and has, for this reason, sometimes been called "the chloroplast reaction" [Hill (1), Whittingham (2)].

However, as was discussed by Allen and by Whatley in this symposium isolated chloroplasts have now been found capable of carrying out two additional photochemical reactions: photosynthetic phosphorylation, represented by equation 2,† and also, as shown by equation 3, the reduction of CO_2 to the level of carbohydrate with an accompanying evolution of O_2 (5).

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† P_i represents orthophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate.

$$\text{Light} + \text{ADP} + P_i \longrightarrow \text{ATP} \quad (2)$$

$$\text{Light} + H_2O + CO_2 \longrightarrow (CH_2O) + O_2 \quad (3)$$

As discussed more extensively elsewhere (3–5) chloroplasts appear in the light of present evidence as remarkably complete and autonomous cytoplasmic structures which contain all the enzyme systems needed for photosynthesis. These enzymes are divided into three main groups, each controlling an increasingly complex phase of photosynthesis: photolysis of water, photosynthetic phosphorylation, and CO_2 fixation. Whole chloroplasts contain all three groups of enzymes. Chloroplasts broken by treatment with water contain only

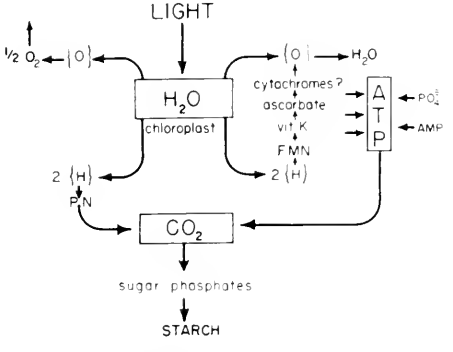

Fig. 1. Scheme for photosynthesis by isolated chloroplasts. Photolysis of water (center) leading either to ATP synthesis and the reconstitution of water (right) or to CO_2 reduction (below) linked to oxygen evolution (left) (4).

two; the group of CO_2-fixing enzymes is leached out. The phosphorylating enzymes, which are not water-soluble, remain bound to the particles, as do the enzymes of photolysis.

It is visualized that *in vivo* photolysis is linked (Fig. 1) either with phosphorylation, resulting in the production of ATP and the reconstitution of water, or with CO_2 fixation, resulting in the evolution of oxygen and the reduction of CO_2 to the level of carbohydrate (equation 3). CO_2 fixation requires the participation of all three groups of enzymes, phosphorylation requires two, whereas photolysis of water can proceed without the others provided an artificial hydrogen acceptor is supplied. The last process, the well-known Hill reaction (equation 1), provides a convenient method for measuring the activity of the enzymes concerned in the photolysis of water, under non-

physiological conditions when neither photosynthetic phosphorylation nor CO_2 fixation takes place.

This postulated increasing order of complexity is supported by the experimental separation of the three chloroplast reactions (equations 1, 2, and 3) by means of differential inhibitors. Chloroplast preparations capable of carrying out complete photosynthesis were used in three parallel series of experiments in which CO_2 fixation, photosynthetic phosphorylation, and photolysis (Hill reaction) were measured separately. It was possible to inhibit a more complex phase of photosynthesis without affecting the simpler one which preceded it and, conversely, inhibition of a simpler phase of photosynthesis was invariably paralleled by an inhibition of the more complex phase which followed it (Table I). Thus iodoacetamide and arsenite inhibited CO_2 fixation but not photosynthetic phosphorylation or the photolysis reaction. Dinitrophenol inhibited photosynthetic phosphorylation and CO_2 fixation more than photolysis. Methylene blue and *p*-chloromercuribenzoate inhibited both CO_2 fixation and photosynthetic phosphorylation but not the photolysis reaction. On the other hand *o*-phenanthroline, which inhibited photolysis, also inhibited photosynthetic phosphorylation and CO_2 fixation. A more detailed discussion of the effects of inhibitors on photosynthesis by chloroplasts is given elsewhere (3).

As shown in Fig. 1, it is envisaged that the recombination of the products of decomposition of water in photosynthetic phosphoryla-

TABLE I. Differential Inhibition of Photolysis, Photosynthetic Phosphorylation, and CO_2 Fixation

Inhibitor	% inhibition of		
	Photolysis	Photosynthetic phosphorylation	CO_2 fixation
10^{-4} *M* *o*-phenanthroline	83	58	73
8×10^{-4} *M* dinitrophenol	50	82	87
2.5×10^{-4} *M* *p*-chloromercuribenzoate	0	85	94
10^{-5} *M* methylene blue	0	93	95
2×10^{-3} *M* arsenite	0	7	99
5×10^{-3} *M* iodoacetamide	0	—	79
10^{-2} *M* iodoacetamide	0	10	—

tion proceeds in several successive steps which together constitute an "electron ladder" analogous to that discussed for respiration by Lipmann (6). The generation of ATP during photosynthesis by a phosphorylation linked to the recombination of the products of photodecomposition of water was first postulated on theoretical grounds by Ruben and has since occupied a position of varying prominence in several schemes of photosynthesis (see review, 7).

Discussion

Strehler: Apropos of your last sentence, there is some evidence in studies on whole cells which is consistent in the main with the conclusions that you have made. When we measured ATP formation in illuminated *Chlorella*, one of the things that concerned us most was whether this was merely an oxidative phosphorylation through the mitochondria, or whether it was directly tied to the photochemical process. We came to the conclusion that at least a goodly portion of the ATP that was formed as the result of illumination was not due to respiratory activity. The conclusion was based upon temperature studies in which we showed (*1*) if oxygen was admitted to an anaerobic preparation of *Chlorella* and the rate of ATP formation was measured at two temperatures, 25°C. and 4°C., there was a large temperature dependence of the reaction. (*2*) On the other hand, if one measured the light-induced ATP formation in the same preparation, one observed a much lower dependence on temperature.

Moreover, as the rate of light-induced phosphorylation at 25°C. was considerably lower than that of the oxygen-induced ATP formation, I think it would be wise to be cautious about the assumption that the only site of important ATP formation for photosynthetic purposes is in the chloroplasts, inasmuch as the rate induced by oxygen at 25°C. is about three or four times that produced by light.

Arnon: As for the last part of your comment, we are inclined to the conclusion that photosynthetic phosphorylation is the dominant kind of phosphorylation in photosynthesis. We base this conclusion on the point that photosynthesis in saturating light is anywhere from 20 to 30 times as high as respiration, and so we do not think that respiration could keep pace with the demands for ATP, and that is the reason why we believe that the cell has a need for an auxiliary abundant supply of ATP which is independent of the respiratory activities of the cell.

Frenkel: In comparing the characteristics of oxidative phosphorylation with those of photosynthetic phosphorylation, are data for oxidative phosphorylation taken from observations on chloroplast fragments or on animal mitochrondria?

Arnon: Our antimycin data for chloroplasts are compared with those by Lehninger, Chance, and others, on the effect of antimycin on animal mitochondria. All the other information, insofar as it pertains to photosynthetic phosphorylation, is our own. Insofar as it pertains to oxidative phosphorylation, it is taken from the literature with the one exception on the comparison of oxidative and photosynthetic phosphorylation between mitochondria and chloroplasts from the same leaves.

Krall: Has the effect of cyanide or carbon monoxide been studied in your system?

Arnon: Cyanide inhibits CO_2 fixation much more than it inhibits photosynthetic phosphorylation, and both of these more than photolysis, which is hardly inhibited at all by cyanide. So there is a differential effect the same as with the other inhibitors. However, I think the results of the cyanide inhibition should be taken with great caution.

Bassham: I just want to point out that Dr. Arnon suggested in his chloroplast preparation there are separate pathways for electron transport to the carbon-reduction cycle and to the phosphorylation system. This is not surprising, since vitamin K can be a Hill oxidant and more or less short-circuit the path of the electrons from the photochemical reaction to photosynthetic phosphorylation. However, since two separate paths are indicated, and since in photosynthesis in whole plants there is probably a different path for the electrons from the photochemical reaction to carbon dioxide reduction from the pathway to phosphorylation here. I don't think one can draw any conclusions from these data regarding the matter of the electron transport system between the photochemical part and the carbon-reduction part.

Arnon: I am sorry but I do not understand the last point. What is the basis for the statement that in the intact cell there would be a different electron transport?

Bassham: It is quite possible that in the intact cell we don't known as yet the path between the photochemical reaction and the reductant that is used in the carbon-reduction cycle. But in your chloroplast preparations you may be short-circuiting this path by putting in vitamin K or FMN, which accepts the electrons directly from the Hill reaction and then uses these for the photosynthetic phosphorylation. If there is such a short-circuit as that, one cannot draw any conclusions regarding the nature of the electron transport system in photosynthesis between the photochemical and the carbon-reduction cycle.

Arnon: We don't regard this as a short-circuit at all.

Bassham: Do you have any reason for not regarding it as a short-circuit?

Brown: It is a basic disagreement—not a matter of misunderstanding.

Arnon: No, there is some misunderstanding which I would like to clear up. We believe that in photosynthesis, at least in photosynthesis by isolated chloroplasts, there are two normal sinks for electrons, one to generate ATP and the other to reduce CO_2. The CO_2 sink is dependent on ATP I was trying to point out (this is the point which is very important in our scheme) that in no case have we ever had CO_2 fixation by a chloroplast preparation which has lost the capacity for ATP formation. We consider that ATP is a prerequisite for CO_2 fixation. Thus the formation of ATP is a normal path in our scheme, not a short-circuit.

The only short-circuit which would occur in our scheme would be the Hill reaction. When we substitute an artificial electron acceptor, such as quinone or ferricyanide, for the phosphorylating pathway, we simply do not permit the electrons to recombine with the oxidant and to carry out the coupled phosphorylation. Instead we get the Hill reaction.

Lucile Smith: I am wondering why you think it is necessary to add vitamin K back to your preparation. Do you think that in the preparation of chloroplasts

the vitamin K is lost, or do you think that it is in some way inactivated? It is very insoluble in water.

Arnon: I think it may be partly inactivated. We have some recent data based on the response to total vitamin K, that is, vitamin K present in the chloroplast plus vitamin K added, and there is good agreement there. We think that perhaps part of the reactive surface may be inactivated in the preparation. That is a possibility.

Clendenning: In your scheme there were two sources for oxygen. Do you find an inhibition of oxygen evolution when ATP is put in the system?

Arnon: We can inhibit CO_2 fixation quite appreciably by putting in the components of the phosphorylating system.

Clendenning: How about oxygen evolution?

Arnon: Which would correspond to the same thing in our scheme of things. We obtain oxygen evolution only when CO_2 fixation takes place.

Whatley: However, in the experiment in question only CO_2 fixation and not oxygen evolution was measured.

Clendenning: What is the importance of the isolation procedure versus the reaction conditions in the oxidation convection system that you have? As far as I can see, the actual isolation procedure has not changed so very much since way back in the nineteenth century. Engelmann and many others in that period knew very well how to isolate whole chloroplasts. It is a very simple matter.

Arnon: Engelmann did not measure photosynthetic phosphorylation.

Clendenning: He knew how to isolate whole chloroplasts. What is added after you isolate the whole chloroplast?

Arnon: Engelmann did not discover the Hill reaction, although he observed oxygen evolution, because he did not add the hydrogen acceptor. His chloroplasts were capable of doing the Hill reaction and probably also capable of doing photosynthetic phosphorylation and CO_2 fixation if he had known what cofactors to add.

Frenkel: Dr. Arnon and Dr. Whatley presented data with regard to the vitamin K requirement of photosynthetic phosphorylation by chloroplast fragments in the presence of *ascorbate*. I was told by Mr. David Geller at Dr. Lipmann's laboratory in Boston that in the partially purified photophosphorylating system from *Rhodospirillum rubrum* vitamin K apparently is not required for full activity when succinate is added. However, when succinate is replaced by ascorbate the addition of vitamin K has a pronounced effect in increasing the rate of photophosphorylation of bacterial preparations over the rate with ascorbate alone. This observation makes me wonder whether vitamin K is required for the phosphorylating system per se, or whether its sole function is to help transfer electrons from ascorbate to the phosphorylating system. It might be of interest to see whether other substances than ascorbate will give rise to active photophosphorylation in the chloroplast system and whether such substances if found will also replace the vitamin K requirement.

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5. *Phosphate Metabolism*

Light-Induced Phosphorylation by Cell-Free Preparations of *Rhodospirillum rubrum**

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Cell-free preparations of the nonsulfur purple bacterium *Rhodospirillum rubrum* when illuminated with white or red light under anaerobic conditions (to avoid complications due to photooxidation (1)) will esterify orthophosphate in the presence of ADP (Fig. 1) (2) which is transformed into ATP in an almost quantitative manner in the presence of a moderate excess of orthophosphate (3). Certain other substances can act as acceptors for the high-energy phosphate formed in the light as listed in Table I, *2*. The rate of phosphorylation is influenced by light intensity as indicated in Fig. 2. All observations reported in Table I refer to experiments performed under anaerobic conditions and at saturating light intensities.

Crude cell-free preparations as indicated in Table I can use the 5′ isomers of AMP, ADP, and IDP as acceptors of the high-energy phosphate formed as a result of the photochemical reaction. The initial rates of phosphate esterification in the presence of any one of these three acceptors do not differ by more than ±5%. Additions of a number of substances (Table I, *4*) have little or no effect on the initial rates of phosphorylation or on the total amount of orthophosphate esterified for a given amount of added phosphate acceptor.

When a crude cell-free preparation is subjected to further differ-

* Abbreviations used: AMP, ADP, ATP for the 5′ isomers of adenosine monophosphate, diphosphate, and triphosphate, respectively; IMP, IDP, ITP for the 5′ isomers of inosinic acid, inosine diphosphate, and triphosphate, respectively; DPN for (oxidized) diphosphopyridine nucleotide.

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ential centrifugation, the material sedimenting at 60,000 × g (for 1 hour) contains most of the phosphorylating activity which can be induced in the light, but this activity can be elicited from a well-

TABLE I. Requirements for Light-Induced Esterification of Orthophosphate by Cell-Free Preparations of *Rhodospirillum rubrum* under Anaerobic Conditions

A. Crude cell-free preparations (Supernatant from sonically disintegrated material after centrifugation for ½ hour at 25,000 × g).	B. Partially purified preparations Crude preparation obtained as indicated under (A), then centrifuged at 60,000 × g for 1 hour. Sediment resuspended in 0.2 *M* glycylglycine at pH 7.5. Centrifuged again at 60,000 × g for 1 hour and resuspended in glycylglycine buffer pH 7.5.
Requirements	
1. Orthophosphate	
2. Acceptors of high-energy phosphate	
Adenosine-5′-phosphate	(Adenosine-5′-phosphate active only with the following additions: trace of ATP; adenylate kinase preparation from *R. rubrum*, present in the supernatant of the crude preparation after centrifugation for 1 hour at 144,000 × g).
Adenosine-5′-diphosphate	
Inosine-5′-diphosphate	
2b. Substances inactive as acceptors of high-energy phosphate produced by the photolytic system	
	Adenosine-5′-phosphate, except with additions as indicated under (*2*)
Adenosine-3′-phosphate (± trace of ADP or ATP)	
Inosine-5′-phosphate (± trace of ADP, ATP, or IDP)	
Uridine-5′-phosphate (± trace of ADP or ATP)	
Creatine (± trace of ADP or ATP)	
3. Additional requirements in the presence of ADP as acceptor of high-energy phosphate	
None observed thus far	Mg^{++} at a concentration of about 5×10^{-3} mole per liter; DPNH, less than 0.01 mole per mole of orthophosphate esterified; or traces of succinate, lactate, etc. (ref. 4)
4. Substances which have little or no effect on the rate of light-induced phosphorylation by crude or partially purified preparations in the presence of ADP and minimum requirements as listed under *3*	

TABLE I (*Continued*)

Substance	Final conc. (moles/liter)	Remarks
Diphosphopyridine nucleotide (DPN)	2.5×10^{-4}	
Triphosphopyridine nucleotide (TPN)	2.5×10^{-4}	
Cytochrome *c* (mammalian)	1.2×10^{-5}	
Cytochrome *c* + DPN	1.2×10^{-5} + 2.5×10^{-4}	
Potassium fluoride (KF)	1.0×10^{-2}	In crude preparations KF slightly reduces the rate of orthophosphate release in the dark. This effect does not influence appreciably the net rate of light-induced phosphorylation at saturating light intensities.
Hydroxylamine (NH_2OH)	0.14	Prepared from $NH_2OH \cdot HCl$ by neutralization with KOH immediately before addition to experimental vessels.
Gas phases other than helium:		
Experimental / Control*		* Control rates do not differ from those in the experimental systems by more than ±5%.
N_2 / He		
5% CO_2 in N_2 / 5% CO_2 in He		
He** / He		** Alkaline pyrogallol in side arm. Shaken in the dark for ½ hour before illumination.
Carbon monoxide (97.3%; remainder H_2 and CO_2)***		*** Rates the same in red light (red filter with cut off at 617 mμ) as in white light (incandescent lamps).

washed preparation only when, in addition to orthophosphate and ADP, a magnesium salt is added and traces of a substance like succinate, lactate, etc. (4), or traces of reduced DPN. It is suggested that these substances activate the system by reducing a component of the electron, or hydrogen, carrier system (C, CH in Fig. 3) which is capable of coupling with a phosphorylating system (P, Fig. 3). It is assumed that in the absence of a reduced carrier (CH) the

photochemical oxidant (OH) will preferentially react back directly with the photochemical reductant (H) (5), this reaction not being accompanied by phosphorylation. However, once electron transport

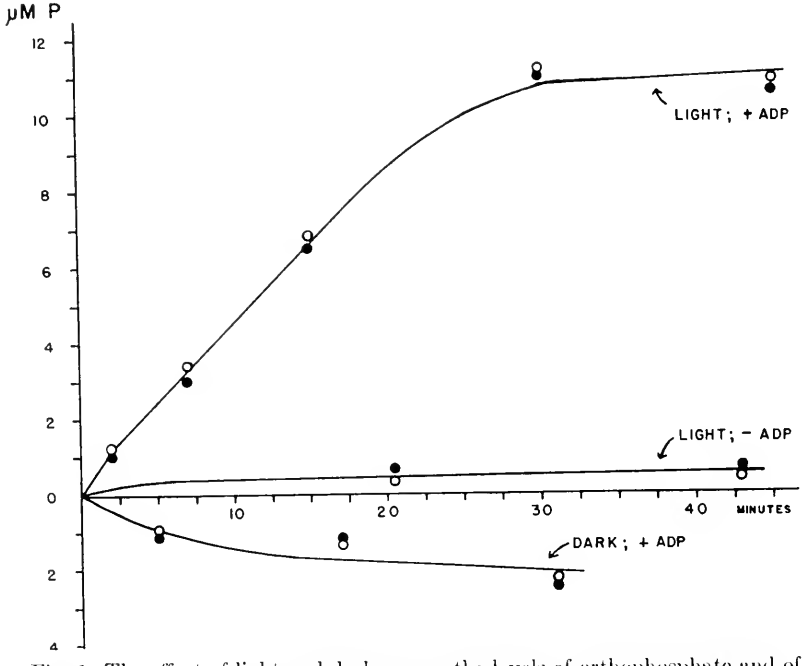

Fig. 1. The effect of light and darkness on the levels of orthophosphate and of labile phosphate (hydrolyzed in 7 minutes by N HCl at 100°C.) in the presence of crude preparations with and without added ADP. (○) orthophosphate taken up, (●) labile phosphate formed. Preparations in 0.2 M glycylglycine (potassium salt) containing 13.4 mg. dry weight protein per vessel with the following additions: 35 μM $MgCl_2$, 30 μM KF, 20 μM orthophosphate, 10.3 μM ADP, 1.5 μM DPN; final volume per vessel 3.0 ml., pH of reaction mixture 7.4. Temperature 25°C. Light intensity 1200 foot-candles (incandescent lamps). Gas phase helium.

has been initiated through the carrier chain which is associated with a phosphorylating system, this pathway can be maintained by alternate oxidation and reduction of the carrier system by (OH) and (H). In addition, there is good evidence that the reducing agent plays a role in protecting the system from inactivation, most likely by photooxidation (1,3b), occurring even at rather low partial pressures of

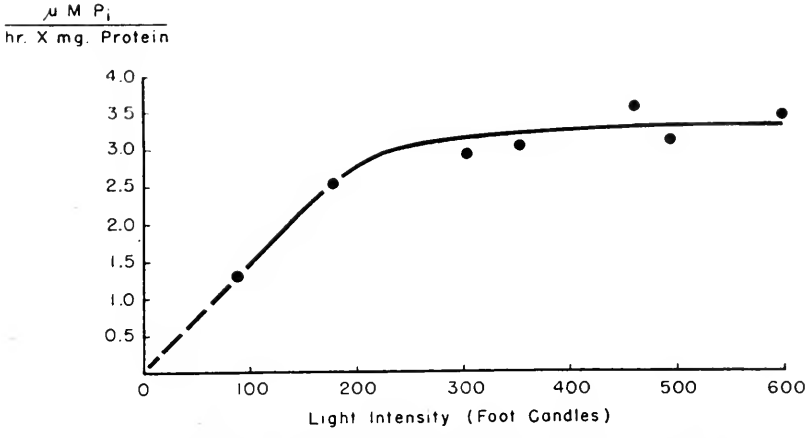

Fig. 2. Initial rates of light-induced phosphorylation as a function of light intensity (against initial blanks). Crude cell-free preparations in 0.2 *M* glycylglycine (potassium salt) containing 15 mg. dry weight protein per vessel with the following additions: 10 μM ADP, and 15 μM orthophosphate. Final volume per vessel 3.0 ml., pH of reaction mixture 7.5. Optical density of suspension 1.5 at 800 mμ. Temperature 25°C. Illumination by incandescent lamps.

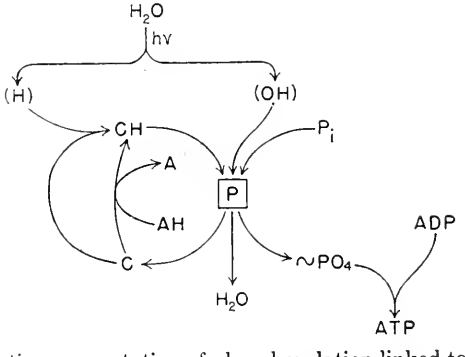

Fig. 3. Schematic representation of phosphorylation linked to a photochemical process (*cf.* ref. 9). (H) photochemically produced reductant; (OH) photochemically produced oxidant; C, CH, oxidized and reduced intermediate carriers; AH, exogenous electron donor ("sparking" agent) reduces C to CH in the dark; [P] phosphorylating system.

oxygen. It is not known at which point along this chain of electron transport phosphorylation occurs, its location in Fig. 3 being quite hypothetical.

It may be of interest that the washed preparations cannot utilize AMP as acceptor of high-energy phosphate (Table I, *2*) although the crude preparations can use it quite effectively. Upon addition of a trace of ATP and of a partially purified adenylate kinase preparation obtained from the same organisms according to the method of Colowick and Kalckar (6), the particles sedimenting at 60,000 × g can again utilize AMP as the acceptor of high-energy phosphate. In contrast to animal mitochondria, where adenylate kinase usually is associated closely with these structural units (7), the photophosphorylating particles described here can be obtained free of such enzymatic activity. There is no evidence for the presence of an enzyme transforming IDP into IMP and ITP, as only IDP is active as acceptor of high-energy phosphate, and IMP is inactive even in the presence of traces of ITP in crude or washed preparations. Other nucleotides besides those listed in Table I may well be able to act as acceptors of the high-energy phosphate produced by the photolytic system.

The reaction described above very likely represents a net conversion of light energy into chemical energy by a cell-free bacterial system, but this assumption will have to be tested, for example, by the calorimetric method described by Arnold (8).

PHOTOPHOSPHORYLATION AND OXIDATIVE PHOSPHORYLATION

When the cells of *Rhodospirillum rubrum* are disintegrated in a sugar solution, cell-free preparations are obtained which will esterify orthophosphate aerobically in the dark. With substrate amounts of alpha ketoglutarate (KGA) one can obtain P/O ratios as high as 1.5, and this phosphorylation is partially sensitive to 10^{-4} *M* dinitrophenol (at pH 7.2) which will lower the P/O ratio to about 0.8. The rate of aerobic dark phosphorylation of these preparations in the presence of KGA is approximately the same as the rate of anaerobic light phosphorylation. The washed photophosphorylating system, on the other hand, shows little or no oxygen uptake aerobically with or without KGA, and phosphorylation cannot be observed in the dark even when hexokinase and mannose are used as trapping agents for ATP. Oxidative phosphorylation by cell-free preparations from *R. rubrum* also has been observed by L. Smith (10) who has evidence that this phosphorylation is associated with particles of a different nature than the particles which carry out light phosphorylation.

The relationship of these two systems is not clear at present and will require further elucidation.

Discussion

Whittingham: Which of the sediments has the highest activity?

Frenkel: The material containing the highest activity roughly has the same sedimentation behavior as the "chromatophores" isolated from *Rhodospirillum rubrum* by Schachman, Pardee, and Stanier.

Lucile Smith: Is oxidized DPN just as effective as reduced DPN?

Frenkel: No, oxidized DPN does not work in the light system studied.

Vishniac: Have you tried the effect of some of the chelating agents which have been shown to enhance oxidative phosphorylation by removal of calcium ions?

Frenkel: I have tested Versene only with the crude preparations and have not observed any significant effects.

Vishniac: The fraction which you believe to be active myokinase—could it be replaced by nucleotides? The reason I ask is because, in the study of oxidative phosphorylation, Pinchot discovered a heat-stable factor which at first he believed to be myokinase, just as you indicated. However, it turned out to be nucleotides.

Frenkel: The heat-stable factor described here is nondialyzable, precipitates with perchloric acid (8%), will convert ADP into AMP + ATP, and also in other ways resembles the myokinase described by Colowick and Kalckar.

Tolbert: Can you estimate whether the chlorophyll/protein ratio in your small particles is the same as that in the whole cell?

Frenkel: No, I have no such analyses. However, I would like to mention an interesting observation. As indicated in ref. 2, when crude preparations are washed by repeated high-speed centrifugations, the photophosphorylating activity based on chlorophyll content remains constant. Thus it appears that in the fractionation procedures employed thus far, the photophosphorylating system has not been separated from the photochemical system and most likely is on one and the same particle.

Newton: We have some observations that I think bear on your hypothesis. Dr. Kamen and I have been studying a similar system in purple sulfur bacteria which I will discuss later, but pertinent here is the fact that the phosphorylation which we observe is inhibited by small amounts of substances like thiosulfate which can serve as hydrogen donors for photosynthesis in these organisms, and it might be that what we are doing is siphoning out the photochemical oxidant with the added hydrogen donor.

Frenkel: I would like to make a comment about the demonstration of a vitamin K requirement. Mr. D. Geller at Dr. Lipmann's laboratory has studied the vitamin K requirement of the photophosphorylating system from *R. rubrum*. As far as I know he has not observed such a requirement for washed preparations in the presence of succinate. However, in the presence of ascorbate the washed system is not activated unless vitamin K is added, and in this way it behaves very much like the chloroplast preparations of Dr. Arnon and co-workers. It may be that, at

least in the bacterial preparations, added vitamin K is not an essential component of the photophosphorylation system; and, only when ascorbic acid is added, does it become a suitable hydrogen carrier. However, reduced vitamin K itself will activate the photophosphorylating system.

Vernon: Have you determined what happens to the succinate? Does it go to fumarate and stop there or is it photometabolized further?

Frenkel: I do not know. I have been told by Mr. D. Geller that fumarate is inactive in this system, and that upon addition of malonate the amount of succinate required to carry out photophosphorylation is extremely small, malonate by itself being inactive. Succinate thus appears to act in a catalytic manner but apparently not by way of succinic dehydrogenase.

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Light-Induced Phosphorylation in Extracts of Purple Sulfur Bacteria*

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We have been studying the electron transport systems which may be peculiar to the photosynthetic process in purple bacteria. The purple sulfur bacterium *Chromatium sp.*, strain D, was selected for study because it provided a system for investigating such processes in an organism which photosynthesizes in the complete absence of a functional aerobic metabolism. In *Chromatium*, growth and photosynthesis are obligately anaerobic processes. Aerobic oxidative processes do not appear to provide the cells with any biochemical energy utilizable for growth. Recently, Dr. A. Frenkel has described a light-induced phosphorylation of ADP to ATP in extracts made from the photoheterotrophe *Rhodospirillum rubrum*. We have been able to extend these observations to extracts made from *Chromatium* and to show that light induces the phosphorylation of ADP under strictly anaerobic conditions. We have succeeded in partially purifying the light phosphorylation system by differential centrifugation. The *Chromatium* extracts are not so active as the *R. rubrum* preparations in carrying out photophosphorylation, but extracts can be prepared which form in the light about 0.5 μm of ATP per hour per milligram protein. In contrast with the *R. rubrum* extracts, *Chromatium* preparations invariably exhibit, in addition to the light effect, a dark phosphorylation which appears to result from the fermentation of endogenous reserves, since it can be reduced by anaerobic incubation of the cells in buffer in the dark prior to preparation of the extracts. Extracts of *Chromatium* prepared by high-speed homogenization with glass beads can be separated into a number of different fractions by differential centrifugation. Centrifugation of crude extracts at 25,000 $\times$ g for 1 hour, sediments the bulk of the pigment containing

* This work is made possible by the continued financial support of the C. F. Kettering Foundation.

particles as "chromatophores"; these will carry out both a dark- and a light-enhanced phosphorylation reaction. The supernate from this initial centrifugation still contains some of the bacteriochlorophyll and carotenoids in smaller particles which can be sedimented by centrifugation at 100,000 × g for 90 minutes, leaving a clear yellow supernate. This shows no light-stimulated phosphorylation but forms some ATP from added ADP in the dark because it contains myokinase activity. The pigmented, small particle fraction sedimented at 100,000 × g cannot carry out the light-induced phosphorylation reaction unless some of the supernatant liquid is added to it. The factor present in the supernate from *Chromatium* extracts which activates the light reaction is a heat-stable dialyzable material, and its effect cannot be duplicated by addition of metals, ashed supernate, ascorbate, menadione, flavin mononucleotide, chelating agents, organic acids, pyridine nucleotides, or sulfur compounds when these are added either singly or in combination to the small particle fraction. So far, nothing which we have investigated will replace the activating effect of the soluble "supernate factor." In fact none of the known materials which we have added will enhance the light effect, although some compounds have shown inhibitory effects. So far the nature of the soluble, heat-stable supernate factor is unknown.

Discussion

Duysens: Did you add DPNH?

Newton: No, we have not added DPNH. We have added DPN in the presence of various organic acids. We have added cytochrome *c* and cytochrome isolated from the organisms. We have not added yeast extracts. (*Note added in proof:* Subsequent studies have shown that yeast extracts do activate the light phosphorylation in the small particles.)

A Light-Reversible Carbon Monoxide Inhibition of Isotopic Phosphate Uptake by Photosynthesizing Barley Leaves*

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The relationship between the respiratory process as it occurs in the darkened plant and the photosynthetic process in that same plant when illuminated is a matter of perennial experimental interest.

The conclusion that oxidative action is probably necessary at least for prevention of induction effects has been drawn by Hill and Whittingham (1) measuring oxygen evolution in *Chlorella* and by Krall and Burris (2) and Krall (3) measuring $C^{14}O_2$ uptake. The latter authors on the basis of inhibition by carbon monoxide suggested that cytochrome oxidase mediated the oxidative reaction involved.

The first work that indicated the existence of carbon monoxide effects on photosynthesis was that of Padoa and Vita (4) where assimilation was inhibited while respiration was stimulated. Gaffron (5) did the first systematic study of the effects of CO on *Chlorella*. He found that fairly long exposures to CO in darkness produced an induction effect more pronounced than that caused by exposure to nitrogen for the same length of time. He observed rates of pressure change following the induction period which were lower in the CO-treated cells than in the nitrogen controls. In some cases the CO effect was completely reversible by exposure to air but in others where exposure to CO in darkness was longer it was irreversible. Gaffron considered mainly two different explanations for the effects he observed: (*1*) inhibition of a photo-catalase, after establishment of rigid anaerobiosis, by CO, which inhibition was reversible by oxygen, or (*2*) formation of inhibitory fermentative products in

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darkness under CO whose removal in light was hindered by the effect of CO on respiratory, nonphotosynthetic reactions.

This paper reports studies on isotopic phosphate incorporation into phosphate esters involved in photosynthesis while the tissue was exposed to air or to gas mixtures containing carbon monoxide under various conditions of illumination. The object of the experiments was to test the hypothesis that the activity of an enzyme analogous to cytochrome oxidase is necessary for photosynthetic esterification of phosphate.

METHODS

P^{32} was incorporated into weighed amounts of first leaves of barley that were 6 to 9 days old by immersing the cut ends of the leaves in a dilute aqueous phosphoric acid solution containing a known amount of the isotope. The P^{32} was taken up in the transpiration stream during a 1- to 2-hour exposure of the leaves to a gas mixture containing the inhibitor or to a control gas. In some experiments the leaves took up the isotope in air under white light for a long period of time (24 to 36 hours) before exposure to inhibitory conditions.

The incorporation of P^{32} was terminated by dropping the leaves into boiling 30% ethanol and extracting for 2 minutes. The extract, after concentration *in vacuo* to 8 to 10 ml., was made alkaline with dilute ammonia and fractionated on a Dowex-1-Cl column by a modification of the method of Khym and Cohn (6), in which a shorter column and continuous recording of the P^{32} in the column effluent permitted much more rapid analysis. The fractions were then assayed for P^{32} with a dipping-tube counter.

The carbon monoxide-carbon dioxide-oxygen mixtures used as inhibitors were made up in 40-liter bottles by water displacement and were forced over the leaves at the rate of 5 to 10 liters per hour during an experiment. The leaves were illuminated with a 150-watt reflector flood lamp giving an intensity of about 2000 foot-candles at the leaves or by the same type of lamp behind a Corning 2030 red filter which gives a very much lower light intensity, probably equivalent to no more than two to three times compensation level of light.

A sodium arc lamp rated at 100 foot-candles was used as a source of light for reversal of the carbon monoxide inhibition. Its effectiveness in promoting photosynthetic uptake of $C^{14}O_2$ had previously been found to be very low (3).

All the experiments reported here have been repeated at least three times. The data reported in the table are those of individual typical experiments.

RESULTS

Table I shows the percentage of elutable P^{32} found in phosphate esters of interest in this study. These figures are a quantitative measure of the isotopic phosphate found in the esters in the plant, since further treatment of the tissue with boiling 30% ethanol extracts gave very little more radioactivity, and since quantitative recovery from the column has been demonstrated for those compounds considered here.

TABLE I. Distribution of P^{32} among Phosphates Separable from Barley Leaves after Exposure to CO

		% P^{32} in Fractions					c./min. × 10^{-3}		
Expt.	Condition	PO_4	HMP	PGA and Triose PO_4	HDP	ATP	Total PO_4	Org PO_4	HMP
1	Air-white 100 min.	51	6.4	21	8.5	5.6	2400	1190	155
2	Air dark 120 min.	85	3.7	2.6	2.6	1.2	540	80	20
	Expts. 3–5: 98.5% CO, 1% O_2, 0.5% CO_2, 100 min.								
3	Red	94	1.8	1.3	1.2	0.13	2000	140	36
4	Red	82	5.5	2.6	5.7	1.1	610	114	34
5	Yellow	77	5.9	5.2	5.1	2.1	420	98	25
	Expts. 6–9: 94.6% CO, 5.0% O_2, 0.4% CO_2, 120 min.								
6	Red	78	8.9	4.6	3.5	0.5	730	164	65
7	Yellow	77	7.3	3.9	2.8	3.1	470	108	35
8	Red & yellow	66	8.7	8.7	5.2	1.8	2300	760	195
9	Dark	85	3.7	2.7	2.6	1.2	470	71	17.5

Barley leaves (0.92 g.) were exposed to 95 μc of P^{32} in 2 ml. of water under the conditions and for the times shown. Counts on the fraction were made with a glass-wall dipping tube counter under standardized conditions. White light from a 150-watt reflector flood lamp (about 2000 foot-candles on leaves); red light, same lamp behind a Corning 2030 red filter; yellow light from a sodium arc lamp of type used for polarimetry.

The numbers in the first two lines of Table I (Expts. 1 and 2) show the usual distribution of phosphate found in barley leaves in air. The leaves in light show about the same distribution of P^{32} at this

time (100 minutes) as other leaves show when exposed to the same conditions for 36 hours, indicating essentially complete equilibration of the phosphate ester pools with the supplied inorganic phosphate. In Expt. 2, however, a much larger P^{32} content was found in both PGA* and HDP both percentage-wise and on a total count basis when the dark exposures lasted as long as 24 to 36 hours. The low absolute amount of isotopic phosphate incorporated was probably a result of lower transpiration in the darkened plant.

The next three lines are data representative of a set of ten experiments done under oxygen tensions of from about 0.25% O_2 to 1% O_2 using either red or yellow illumination. No significant variation was found in P^{32} distribution under the same illumination over this range of oxygen partial pressure. The differences under the two lights were significant. The mean value for the per cent P^{32} in ATP was 0.75 in red illuminated leaves and 1.86 in those under yellow light. The difference, 1.11%, had a standard deviation of 0.28% and was shown by the t test to be significant at the 1% probability level. In these same ten experiments the sugar monophosphates contained on the average 1.5 times as much P^{32} in the red as in the yellow illuminated leaves. No significant differences were observed in the P^{32} content of the PGA or hexose diphosphate fractions, nor did the total P^{32} esterified by the tissue under the two conditions show a significant difference.

An experiment was done in which a batch of leaves was exposed to P^{32} for 4 hours in air in white light and to air for 12 hours in darkness. A control lot of leaves was extracted without exposure to inhibitor or light. Another lot was exposed to yellow light under CO containing 0.4% CO_2 and 1% O_2. A third lot was exposed to white light under this gas mixture. Both were inactivated after 70 minutes exposure to the inhibitor. In the presence of the inhibitor neither the white nor yellow light seemed effective in increasing the organic phosphate level above that formed in darkness in air. Rather, a sharp drop in ATP level was seen on exposure to CO under either quality of illumination.

The last four lines in Table I show the results of exposing the leaves to a CO mixture containing 5% oxygen under four different con-

* Abbreviations used are PGA for 2- or 3-phosphoglyceric acid, HDP for hexose diphosphate, ATP for adenosine triphosphate, HMP for hexose monophosphate, and triose PO_4 for 3-phosphoglyceraldehyde.

ditions of illumination. The darkened leaves (Expt. 9) showed a P^{32} distribution almost identical with that found if the leaves take up P^{32} in air in darkness, indicating that CO does not interfere with the dark exchange process. These leaves are about the same age (7 to 9 days) as those in which Daly and Brown (7) found no inhibition of respiration by carbon monoxide.

Under red light (Expt. 6), about twice as much P^{32} was incorporated into sugar phosphates and phosphoglycerate as in the same experiments (3 and 4) done under 1% oxygen or less, but the ATP was not labeled as heavily as in the experiments at low PO_2. Under yellow illumination (Expt. 7) the ATP^{32} level approached that found in photosynthesis in air, but the P^{32} in sugar phosphates was still low. The tissue illuminated with both red and yellow light (Expt. 8) showed the most striking changes. All fractions contained more P^{32} than corresponding fractions from leaves exposed to any other condition than air in light. That this increase was not caused simply by greater translocation of P^{32} into the leaf is shown by comparison with Expt. 3 under red illumination at 1% oxygen where almost as much P^{32} was accumulated by the leaf as in air while illuminated with white light, whereas no more was esterified than in other experiments in that series (Expts. 4 and 5) where much less inorganic P^{32} was taken into the leaf.

Probably the best measure of the extent to which yellow and red illumination together were able to stimulate phosphate exchange under CO was found in the total counts P^{32} esterified, a figure which was from five to ten times as high under this combination as under other illumination used in these experiments. The pattern was still not identical with that found in air. The outstanding difference between Expts. 1 and 8 was in the very much higher P^{32} content of the phosphoglyceric acid-triose phosphate fraction in Expt. 1. The ratio of inorganic PO_4/organic PO_4 in Expt. 8 was still twice that found in Expt. 1. Whether this difference was caused by the presence of CO or by the lower than normal oxygen tension as suggested by results of Daly and Brown (7), who found a 50% inhibition of respiration in 8-day barley leaves under 5% oxygen in nitrogen, has not yet been determined.

The last column in Table I compares the P^{32} content of the sugar monophosphates in the leaves in the experiments reported here. As was stated earlier the organic phosphates in Expt. 1 were thought

to be almost at equilibrium with the P^{32} in the inorganic phosphate pool since longer experiments give essentially the same distribution of P^{32} among the various separable components. The esters in the inhibited tissue were not at equilibrium with the inorganic P^{32}, since longer time experiments gave very much higher P^{32} levels in phosphoglycerate and hexose diphosphate and certain as yet unidentified fractions which were isolated by the separation method. Only ATP seemed to possess a constant P^{32} content upon continued exposure to the label under the same conditions. Therefore "% P^{32} in ATP" is used here as a measure of ATP content of the tissue while "counts per minute P^{32} in hexose monophosphate" is used as an approximate measure of hexose monophosphate synthesis. The leaves illuminated by both red and yellow light were the only ones under carbon monoxide which were labeled with P^{32} at approximately a normal rate. If the effects of red and yellow light above that of darkness were additive, one would expect about 80×10^3 counts P^{32} in the HMP—instead there were 195×10^1 counts per minute. Thus illumination with the two lights together has a greater effect in stimulating P^{32} incorporation into organic esters of phosphorus than the arithmetic sum of the effects of the two lights applied separately.

DISCUSSION AND CONCLUSIONS

The observations to be explained are these: Exposure of barley leaves to carbon monoxide containing small amounts of oxygen allows only minimal incorporation of P^{32} into organic esters of phosphate under either the red or yellow lights used. The rate of HMP synthesis was higher in red than yellow light and the ATP level was lower. As long as the PO_2 was low, white light, which may be looked upon as including both red and yellow wavelengths, was no more effective than the yellow light alone in raising the P^{32} level in the organic phosphates above that found in the darkened controls.

At higher oxygen levels the ATP content was almost normal under the yellow light, but P^{32} incorporation into hexose phosphates was still very slow. Under red light more P^{32} was found in the hexose phosphates but the ATP level was only 40% of that found in the darkened inhibited plant. Illumination with a combination of red and yellow light at 5% oxygen gave the nearest approximation, under CO, to the phosphate distribution pattern found in tissue photosynthesizing in air under bright white light.

These observations can be rationalized by assigning the site of CO sensitivity to the enzymatic processes leading to ATP formation. This site could well be a cytochrome oxidase, since its inhibition is reversed by yellow light which is shown here to be effective in raising the ATP level without driving P^{32} labeling of hexose phosphates very rapidly. Red light by itself was also ineffective in promoting phosphate esterification even with 5% oxygen in the CO since the red light cannot reverse the inhibition but can only generate the reductant necessary to convert phosphoglycerate to triose and hexose phosphates. The experiments reported here were long enough so that the oxidase may be said to be participating in a steady-state process rather than in an induction effect.

It is possible that the lowered levels of sugar phosphates were a result of a rapid fermentation under CO. This seems unlikely since, in rapid fermentation, phosphorylation of glucose would be expected, giving higher levels of P^{32} in the HMP fraction.

Thus we favor an explanation which differs from those advanced by Gaffron in that we believe the effects observed to be neither a direct inhibition of oxygen evolution nor an indirect effect by accumulated products of anaerobiosis but the result of a light-reversible inhibition of a cytochrome oxidase involved in photosynthesis. Our main reason for this is that yellow light of a wavelength known to be very effective in dissociating the CO-cytochrome oxidase complex raises only the ATP level and that red light of wavelength ineffective in this process, but effective in photosynthesis, does not raise this level but lowers it—a phenomenon most readily explained by saying that photosynthetic reductant can be utilized by triose phosphate dehydrogenase in forming triose and consumes all the available ATP in the coupled phosphorylation of phosphoglycerate. This lack of ATP then inhibits further triose formation, which inhibition can be relieved indirectly by exposure to yellow light.

SUMMARY

Exposure to CO lowers the amount of P^{32} incorporated by illuminated barley leaves almost to the level which obtains in darkness. Illumination with red light lowers the ATP level in the presence of CO below the level in the dark; illumination by yellow light raises the ATP to the control (air-darkness) level. Only simultaneous illumination with both red and yellow light in the presence of a CO

mixture containing 5% oxygen gives a rate of P^{32} esterification approaching that found under normal photosynthetic conditions in air and white light. Participation of a cytochrome oxidase in the esterification reaction is postulated.

Acknowledgment. The advice and assistance of Doctors A. H. Brown and E. Tolbert concerning preparation of this manuscript is gratefully acknowledged.

Discussion

Whittingham: How long was the system illuminated?

Krall: I worked with whole barley leaves and illuminated the plant in air with 2,000 footcandles of white light from a 150-watt reflector flood lamp for two hours. This is a control experiment with four other experiments which you will see on subsequent slides.

Vernon: I would like to ask Dr. Smith if she has any evidence that this yellow pigment, or the carbon monoxide binding pigment, might be active in the other terminal oxygen system involved in the oxidation of succinate in the chromatophores. In other words, do these oxidize the carbon monoxide binding pigment?

Lucile Smith: No, I don't have any evidence on that. The thing that is definite is that the yellow pigment as now isolated is not capable of oxidizing reduced *R. rubrum* cytochrome c_2. It is not the cytochrome c_2 oxidase.

Chance: What was the effect of oxygen tension on the degree of CO inhibition?

Krall: There was no reversal in the absence of oxygen and practically no reversal with only 1 per cent oxygen. Five per cent oxygen achieved what might be called about a 40 per cent reversal, at least not complete reversal.

Chance: Are there enough data to demonstrate what one would call O_2–CO competition?

Krall: The evidence indicates that it may exist.

Chance: Did you get a partition coefficient?

Krall: No.

Chance: If you could get an oxygen carbon monoxide partition coefficient for this inhibition, then you would really tie it down as involving oxygen. The way it is now it seems to me that the inhibiting effect of the CO could or could not involve oxygen.

Krall: I have studied the effect of CO on $C^{14}O_2$ uptake. 19:1 and 9:1 ratios showed the right competition coefficient. There was about a 50 per cent inhibition at 9:1 and about 95 per cent inhibition at 19:1.

Chance: The second point is that the very high CO/O_2 ratios would give you very little photo-dissociation of the carbon monoxide compound.

Krall: That is why there is little reversal at 1 per cent oxygen. At ratios as high as 400:1 carbon monoxide to oxygen, there was no reversal at all.

Arnon: You interpret all the effects of carbon monoxide as having been limited to cytochrome oxidase. Is this justified in this complex system? Granted that we know what carbon monoxide will do and assuming that those conditions that Dr.

Chance speaks of are satisfied, how can we be sure that we don't have other effects in a system like that?

Krall: One should run an action spectrum. However the yellow light from a sodium lamp is practically monochromatic, and acts on this system the same as white light for reversal under low oxygen pressures. This indicates that there is not an odd cytochrome oxidase that would have a photodissociation spectrum different from the cytochrome oxidase in animal systems.

Arnon: My point goes beyond that. My point really is, how can you ever interpret the effect of an inhibitor, such as carbon monoxide or any other inhibitor, on the whole leaf in terms of a single process?

Krall: The only way I can answer that is to quote W. O. James' article on respiratory inhibitors in "Annual Reviews of Plant Physiology." He states that reversal of carbon monoxide inhibition by light of a specific wavelength is probably the most elegant test we have *in vivo* of testing for an enzyme activity.

Chance: Now you are talking against the man you are working for, Dan!

Gaffron: First, photosynthesis and carbon monoxide in this sort of experiment have a long history. One can read in Rabinowitch's book about some Italians who claimed that photosynthesis is inhibited by carbon monoxide. Many people have tried but could not repeat their experiments. On account of my own experiments with carbon monoxide, I came to the conclusion that if one does anaerobic experiments he has to be sure to exclude a round-about way of inhibition. If one puts aerobic plants into nitrogen or carbon monoxide they don't like it, which is expressed by the fact that after a while the subsequent photosynthesis is seen to be inhibited. Long anaerobic dark periods cause long induction periods for photosynthesis. Quick, yet complete, removal of oxygen does not produce an inhibition. Its cause is, therefore, not lack of oxygen as such but a consequence of anaerobiosis.

If this inhibition is relieved by oxygen, then we may say it is a respiration which does away with the inhibition. By using carbon monoxide instead of nitrogen or helium one prevents this function of respiration. The inhibition persists and one can shine as much red light on it as one wants. If, however, a wavelength is used which is very effective for the release of the carbon monoxide inhibition of respiration, then photosynthesis has a chance to start again.

This was one explanation I discussed at the time, but I added that sometimes I saw an effect of carbon monoxide which cannot quite be explained away in this round-about manner; for instance, its persistence in the presence of some oxygen. A direct inhibition by CO of a "photocatalase," that is of an iron-porphyrin enzyme in the oxygen-evolving systems was the alternative hypothesis. Now, 15 or 20 years later, we may have learned something. I have nothing against the theory that cytochromes play a role in photosynthesis and that this role may consist in creating energy-rich phosphate bonds by back reactions.

The question now is, do you believe in a direct carbon monoxide inhibition of photosynthesis? I think we don't have enough experiments.

Whittingham: Dr. Krall, have you any data on the time course of the phosphorylation with or without CO?

Krall: Very little.

Whittingham: In observations in the light, on phosphate fixation, did you attain

a steady state before you turned your lights off? How much of the observation was in the induction state and how much of it was in the steady state?

Krall: The P^{32} experiments were steady state ones in that they were two hours long. In other cases P^{32} was placed in the plant for a long time, then the plant was exposed to inhibitory conditions and the change in the P^{32} distribution was noted. The same results are obtained either way. The ATP level is very low in the presence of red light, and slightly higher in the presence of yellow light. If you keep the oxygen in, the ATP stays high.

Rabinowitch: My general remark is that when trying to cover the whole field of photosynthesis, as I did, one is always impressed by how often people don't care for work that others have done in this field, particularly if it was done by people who had a different approach and came from a different side. The physical chemists and the physicists are often told that they don't care enough for what the biochemists know about likely and unlikely chemical processes. I think this criticism should have reciprocal validity, and that the biochemists, too, have a certain obligation to look into, not ignore, what has been found by people who approach a problem from the non-biochemical side. This alleged oxygen effect is a case in point.

I must say the suggestion thrown out by Dr. Kamen that the energy of chlorophyll could be transferred to cytochrome is another one. He said something about the need for such a transfer of an absorption band on the red side. But then he said that this is perhaps not so important. I was waiting for Dr. Duysens to protest, but since he did not, I will.

How can one dismiss so lightly the empirical conclusion (which also has a sound physical basis), that energy transfer always occurs towards the longest wavelengths? How can one lightly suggest that the cytochromes may perhaps have a band somewhere in the red, and then add that if they don't have such a band, well, it isn't so important for the resonance transfer anyhow?

Kamen: I did not intend to give the impression I was talking only about cytochromes. I was talking about hemo-proteins in general. There are hemo-proteins here which have not been accounted for yet, which can have the necessary absorption characteristics.

The point is that if you take out the cytochrome and you look at the spectrum of the pure compound, even if you don't find it, it might still be possible that the cytochrome in association with something in the cell can have the proper absorption spectrum.

I don't think that we can argue about the physical chemistry of the chlorophyll molecule from the standpoint of simple magnesium porphyrins. We have to talk about the whole system.

Also I don't have a stake in the cytochrome picture. I simply say we have to consider the possibility of direct excitation of the cytochrome. Whether it is possible on the basis of our present knowledge I don't know.

Gaffron: Would it not be more practical to argue from the things that we know than from the things we don't know?

Kamen: I believe I can say with some conviction that I have heard more arguments from what we don't know from the standpoint of the physical chemists than I have from the biochemists. That is my own feeling about it.

Weigl: Perhaps what Dr. Kamen means is simply that he won't insist on reso-

nant energy, transfer of energy from the chlorophyll to the cytochrome, but rather perhaps a charge transfer or a hydrogen transfer or something else, and that this is the reason he didn't insist on the band.

Rosenberg: I am reminded of a paper by Emerson and Lewis. They pointed out how the quantum efficiency of photosynthesis falls off very sharply beyond the red peak of chlorophyll towards longer wavelengths. At a wavelength above 680 the quanta absorbed do practically nothing for photosynthesis. If there were such cytochrome pigments in large amounts in the chloroplast, perhaps they would absorb a big part of the energy in that region which turns out to be very useless for photosynthetic activity.

Duysens: May I reply to this? This decrease of quantum yield in the deep red region parallels the decrease of fluorescence yield of chlorophyll in the same region, and the same phenomenon occurs in solution. So it is the property of the pigment and not of other absorbing substances in the plant. We find this in all kinds of fluorescent substances.

Smith: Isn't it true that when you go beyond that band you cannot excite the fluorescence any more?

Duysens: You can excite fluorescence, but the fluorescence yield drops.

Brown: Regardless of the anomalies in this region, I wonder if I could ask a very naive question. Maybe some physical chemist can answer it from known fact. If you have an exciton mechanism for energy transfer is it not by definition a primary light absorber: The exciton mechanism that Dr. Kamen is suggesting should extend the action spectrum of photosynthesis out into the infrared.

Allen: Can anybody really say what an exciton is? Perhaps we can start a little farther back.

Rabinowitch: This is a matter of definition. The word "exciton" has been used in two different senses. One of them is identical with resonance transfer of excitation, a system in which energy can be transferred, by resonance, from one molecule to another, staying with each molecule long enough for it to preserve its individuality as an absorber.

However, a more accepted usage now is to speak of exciton when we have such strong interaction between the molecules that they are acting in absorption as a single large super-molecule, in this case, the whole system is different from that of the individual molecule. There is definitely no such exciton present in the cell.

I would also like to mention, in connection with this drop-off in the far red, that Vavilow has received the Stalin prize for the explanation of this phenomenon as a general consequence of thermodynamics, but according to Pringsheim his explanation was wrong.

I think we still don't have a satisfactory explanation, although it obviously is not a specific property of one pigment, but a general property. Of course, one could explain it by saying if an anti-Stokes reaction occurred with a 100 per cent yield, we would have a machine that does nothing but convert smaller quanta into larger quanta, taking energy out of the heat vibrations, which seems to be against the second law of thermodynamics. But this is not a satisfactory proof, since the law could be satisfied by a lowering of the yield below 100 per cent. This is one case in which the physical chemists should hang their heads in shame.

Wassink: What I got from the Emerson-Lewis article is that the absorption is

there but there is no excitation so that you get the absorption but not a fluorescence yield?

Emerson: When we did this work we thought we had abundant evidence that the absorption was there and photosynthesis was not. But I must say that I plan to look at this again and under better conditions. The absorption was dropping very rapidly and it was necessary to take increasing quantities of cells to maintain approximately the totality of the absorption as we went further into the infrared. I still think that the yield drops very rapidly in this region. Just how it drops I think is subject to modifications.

Wassink: Well, my original intention was to make a comment on the dark inactivation of photosynthesis. I think we long ago produced considerable evidence that there is an effect of oxygen on non-photochemical parts of the system. You can demonstrate that for instance by making repeated exposures, say, of 5 or 10 seconds. We have done that with diatoms and with *Chlorella*. We have published it for diatoms not for *Chlorella*, but it is essentially the same.

After the short dark periods the quantum efficiency is the same as in continuous light, whereas the saturation level is much reduced. The latter is reached at lower light intensities. It is restored to the normal level in a period of something over 10 seconds. Also we found that it is connected with a drop in the fluorescence yield which is due to a shift toward the more oxidized condition. So it is very likely that, indeed, oxygen, or at least oxidized compounds, are responsible for restoration of full activity. I mean this is the same as you envisage.

I don't know, of course, how CO comes in there. I think the CO effect overlaps this and inactivates the thing completely in the absence of light.

Smith: Dr. Yocum many years ago found that carbon monoxide inhibits chlorophyll accumulation, not the transformation of protochlorophyll to chlorophyll, but the making of more chlorophyll after the initial transformation. I don't know how this fits in.

Granick: I should like to make a comment on Kamen's suggestion that cytochrome apparently needs some kind of an absorption band a little further toward the red than chlorophyll. We do know of such a thing. A long time ago, Dr. McKalish with a Beckman photometer found bands in the infrared for various substances like sodium iodide, sodium chloride, and water. Testing substances like hemoglobin he picked up what was a small band, it is true, somewhere around 700. That was for oxyhemoglobin, and he tried methemoglobin, fluoride, and a few others. There are very thin bands out in that region. The physical chemists call them forbidden bands. Whether they are forbidden or not they are there.

The question arises whether traces of absorption of this character might possibly be used, say, in the transfer of energy from one pigment to another. I don't know, but it is very interesting to know that they are there.

Strehler: First, in defense of Dr. Kamen, I don't think that it is absolutely necessary that any energy receiving pigments, e.g., cytochromes, have an absorption band in the region beyond or overlapping chlorophyll fluorescence if they are bound to or closely associated with a chlorophyll molecule. In this case the combination of the two pigment molecules might well shift the chlorophyll absorption band to the red far enough that the complex might itself be a sink for light energy through sensitized transfer. Then, since the cytochrome itself would be in direct

association with that particular chlorophyll molecule, it still would have a good chance to be involved in the photochemistry.

Second, I want to ask Dr. Arnon whether he has any evidence of any CO effect with chloroplasts.

Arnon: We have not tried any carbon monoxide effects with chloroplasts. We have a system in which chloroplasts under anaerobic conditions generate ATP at the expense of light energy, but do nothing in the dark. The chloroplast system forms ATP, and reduces CO_2 to sugar. Thus, insofar as photosynthesis by chloroplasts is concerned, we reach the conclusion that it is independent of oxygen.

Krall: But it does this only in the presence of ascorbic acid and riboflavine?

Arnon: Phosphorylation is done also in the presence of menadione or other vitamin K substances. Incidentally, chloroplast is the only natural source of vitamin K_1, and that is the reason we have been looking for a function for vitamin K. Ascorbic acid has also been associated with chloroplasts. There is extensive literature on this subject, going back many years to the Molisch reaction. Flavin is also present in chloroplasts in large amounts. So in answering your question, I am adding the implication that these cofactors are not extraneous substances and that we are observing no artifacts but effects which presumably play a part in these reactions *in vivo*.

Krall: When you first isolated your photosynthetic phosphorylation reaction didn't you show an oxygen requirement before you added these things?

Arnon: Yes, that is so, but that was before we knew about the cofactors.

Rabinowitch: With regard to Dr. Granick's suggestion as to whether a weak band can be used in the transfer of energy from one pigment to another, the answer is that the probability of transfer is proportional to the probability of transition. If the transition is extremely weak, the transition is of a different type, it is a vibrational transition; or if the transition is micronic, that is, a very weak band, the probability of transfer is correspondingly low. It goes to zero when the intensity goes to zero and the state is nonexistent.

Then to answer Dr. Strehler's remarks, of course, you can use light quantities absorbed by chlorophyll molecules in order to produce final transformations in nonchlorophyll molecules. After all, that is what photosynthesis is about. So there is no doubt, for example, that you could use energy absorbed by chlorophyll in order to do something to cytochromes, but not by the resonance energy transfer mechanism.

Strehler: I thought that was what Dr. Kamen meant when he made that statement.

Rabinowitch: Nobody has any objection to saying that instead of reducing carbon dioxide it reduces cytochrome *f*. One is as possible as the other.

[See also Discussion following paper by M. D. Kamen, p. 162.]

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Isolation of a Possible Primary Hydrogen Acceptor from Photosynthesizing Barley Leaves*

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The identity of the primary acceptor for the electrons generated by the photolysis of water in a photosynthesizing system has not yet been established for any *in vivo* system. The most important point established by Hill was that the isolated chloroplast must be given a continuous supply of an exogenous electron acceptor before it can evolve the oxygen at an appreciable rate. Until now, no naturally occurring oxidant, not even diphosphopyridine nucleotide in the closely coupled glutathione reductase system (3), is capable of promoting oxygen evolution at better than 5% of the rate at which the same quantity of chlorophyll can promote its evolution in the whole cell under optimum conditions. Calvin has suggested that 6,8-thioctic acid could act to accept both the hydroxyl radical and the electron formed on photolysis, and (1) shown some stimulation of quinone reduction by algae after preincubation with thioctic acid.

In our studies on nitrogen and carbon monoxide inhibitions of P^{32} uptake by barley leaves, an unidentified phosphate ester was encountered, whose variations with changes in conditions to which the leaves are subjected make it a good candidate for the title of "*in vivo* Hill oxidant." This material apparently exists in the plant in two forms that are rapidly converted from one to the other by changes in illumination and in the redox potential of the external environment. By the anion-exchange chromatography method (4), modified to analyze P^{32}-labeled extracts, the unidentified material is eluted by 0.03*M* NH_4Cl. The application of this eluting agent causes a drop in the pH of the column effluent from 8.2 to 6.5. The bottom curve on Fig. 1 shows the P^{32} level in the liquid during the course of this change, when both components are present. The lower pH level is to the left. The

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pH is about 7.0 at the lowest part of the dip near the center of the eluting curve. The compound on the right has been designated "5a" and that on the left "5b"; 5a is believed to be the reduced form of 5b.

Changes in level of these materials respond to changes in external conditions more rapidly than does the level of any other phosphate

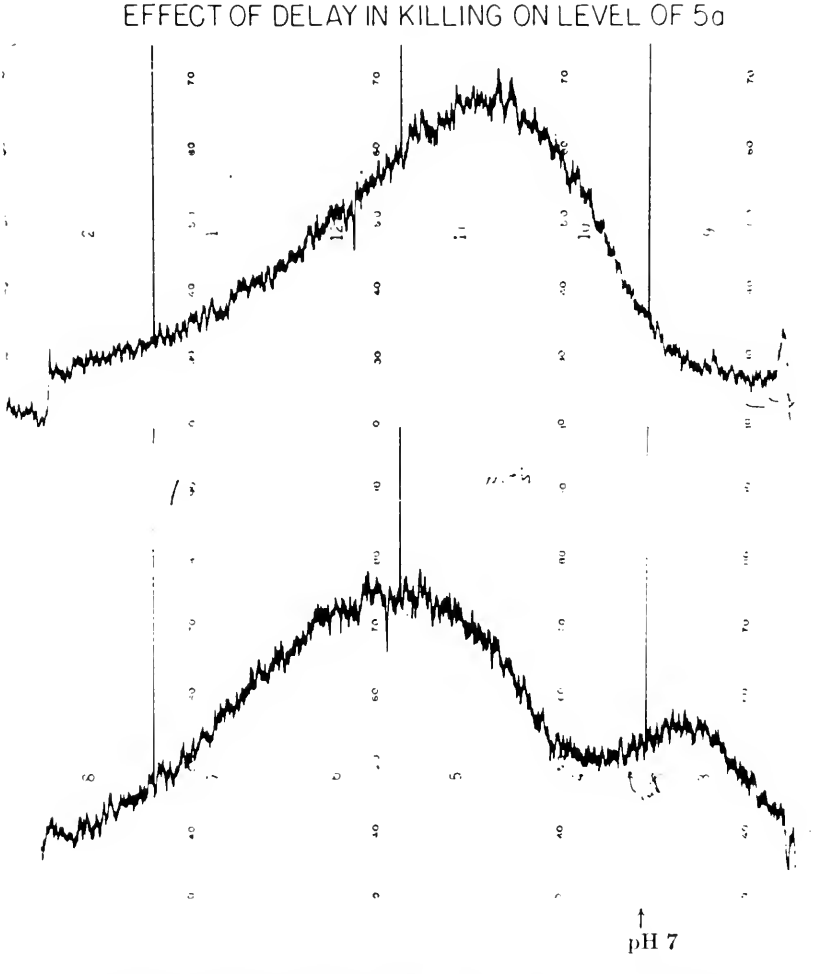

Fig. 1. Recorder traces illustrating separation of 5a and 5b. Increasing eluant volume is to left. Upper trace darkened tissue—expt. 2, Table I; lower trace—expt. 1, Table I. See text for complete explanation.

ester recognizable by this separation. The top trace in Fig. 1 represents a separation in which the leaves were darkened for a short time before extraction. Darkening for as short an interval as 2 or 3 seconds is sufficient to produce the difference exhibited between the upper and lower traces.

Table I illustrates the changes in ratio these substances undergo under different conditions. That 5a is directly related in level to the intensity of the illumination is illustrated by the first five experiments—the brighter the illumination, the higher the level of 5a over a very wide range of intensities. It is not yet known over what range, if any, the relationship is linear. Exposure to two G.E. #50 flash bulbs gave about twice as high a level as exposure to one. These bulbs deliver 110,000-lumen seconds of light in about 50 milliseconds. Thus it is not necessary for the illumination to be spread over a long period of time to produce the unknown, 5a. These experiments were done by triggering the bulb at the same time as the leaves were thrust into boiling ethanol.

TABLE I. Ratio of 5a (Reduced) to 5b (Oxidized) Extractable after Exposure of Barley Leaves to Different Conditions

Condition	Ratio
Air, light 2000 ft-c	1:10
Air, light 2000 ft-c, 2 sec dark	0:10
Air, light 10,000 ft-c	2:10
Air, light 1 #22 flash bulb	5:10
Air, light 2 #50 flash bulbs	15:10
Air, dark	0:1
N_2, dark	1:0
Vacuum, 2000-ft-c, 30 sec	5:10
N_2, 2000 ft-c, 5 min	10:10
CO-CO_2, 1000-ft-c, red 15 min	15:10
CO-CO_2, 1000 ft-c, red 2 hr	$^1/_2$:$^1/_2$
CO-CO_2, 1000 ft-c, yellow 15 min	$^1/_2$:10

Note: Leaves were exposed to P^{32} for 24–36 hr in a light-dark-light sequence before exposure to the above conditions. Figures in the ratio represent the approximate P^{32} content of each fraction obtained by inspection of the recorder trace. The first two experiments are those represented in the figure.

If the light level remains the same and the plant is exposed to anaerobic conditions, the build-up of 5a is rapid. Subjection of the leaf to a 30-second evacuation results in production of 5a in about a third of the maximum attainable amount. Five minutes under nitro-

gen in light or under carbon monoxide in red light gives twice this amount. Raising the intensity of the white light under N_2 gives still more of the material. If, however, exposure to carbon monoxide continues for $^1/_2$ hour or more, the levels of 5a and 5b drop off sharply with 5b dropping first. This decline occurs more quickly with carbon monoxide than with nitrogen and occurs only under those conditions which were found earlier (5) to bring about a drop in ability of the tissue to fix carbon dioxide. It should be noted that exposure of the tissue to carbon monoxide in yellow light affects the tissue no differently from exposure to white light in air.

The presence of these materials is not completely dependent on light, e.g., low levels of 5b are observed under dark aerobic conditions and low levels of 5a under dark anaerobiosis. It is not yet certain whether 5a and 5b depend on carbon dioxide uptake for their maintenance. It is probable that they do not since they take up C^{14} very slowly and only under conditions of net growth. Also the level of neither compound drops rapidly when placed under CO_2-free nitrogen in light, whereas those of photosynthetic products, e.g., sugar phosphates, show a fairly rapid decline.

These data indicate that 5a is convertible to 5b by a dark reaction. Since the 5a piles up under anaerobic conditions, the reaction must be oxidative and 5a more reduced than 5b. The close parallel between the level of 5a and intensity of illumination indicates a close relation between formation of 5a and light. The short time required to produce high levels—less than 50 milliseconds—indicates an extremely close, perhaps direct, relation. The comparatively rapid loss of both 5a and 5b on cessation of oxidation indicates that some product of oxidation, probably high-energy phosphate bonds, is necessary for their maintenance. Nothing has yet been observed which would be incompatible with the hypothesis that 5a is an acceptor in the photolysis of water and is converted to 5b by an oxidative, CO-inhibited process.

What, chemically, are these compounds? It is possible to label them with P^{32}, C^{14}, and S^{35}. The process of labeling is slow with P^{32} and still slower with S^{35} and C^{14}. The last two are incorporated into 5a and 5b in *Chlorella* only in growing cell suspensions. Thus the compound *in vivo* is a relatively stable one as far as C^{14} and S^{35} are concerned. Chromatographic and spectrophotometric analysis of 5a and 5b have shown that they are not pyridine nucleotides, flavins, menadione, or ascorbic acid. The 5b area contains more than one compound: the

P^{32} in this area has been shown to cochromatograph partly with uridylic, cytidylic, and adenylic acids, the first one being quantitatively most important. Some of the label is found in a separate fraction when chromatographed by a formate-Dowex 1 procedure designed to separate the mononucleotides of nucleic acids. This unknown, believed to be the part of 5b which is convertible to 5a, has no 260-mμ absorption, but does have absorption below 240 mμ.

The 5a area contains but one compound. This was ascertained by paper chromatography, wherein all the isotopes incorporated into the material—phosphorus, carbon, and sulfur—move to but one spot on the paper. The R_f value of this spot is about 0.4 in the phenol-water solvent and 0.1 in the butanol-propionic acid developer. 5a gives a positive reaction with the iodine-azide reagent, indicating presence of an —SH group or "masked" —SH. Disulfide bonds do not react with this reagent. 5b does not react with the reagent although it does contain sulfur, which moves to the same spot on paper as does 5a. So far, no positive test has been obtained for thioctic acid by the manometric assay of (2) but this may be the result of too low a concentration rather than absence of the thioctic acid.

In conclusion, two forms of a compound believed to be closely related to, if not identical with, the primary electron acceptor for those electrons generated by photolysis of water in the whole green plant have been isolated from barley, corn, tobacco, and *Chlorella*. Its variations in the plant under changes in physiological conditions are compatible with this interpretation of its function. The compound has been labeled, in *Chlorella*, with radioactive carbon, phosphorus, and sulfur. Only the "reduced" form of the material gives a positive reaction to a test for —SH groups. The compound as yet has not been proved to be identical, in any way, with any known biological oxidation-reduction carrier.

Discussion

Gibbs: Did you ever follow coenzyme I and coenzyme II at the same time that you were following 5a and 5b?

Krall: Coenzyme I and II are eluted at a much different place on the columns than 5a and 5b.

Gibbs: Are there changes in amount of P^{32} in the pyridine nucleotides under these conditions which would correspond to changes in 5a and 5b?

Krall: No, they do not show such changes. In fact there is not a good coenzyme I and II peak. They would come off in eluting agent 6, if present.

Gibbs: Do they change in concentration with light?

Krall: If they do, the change is so small that it has not been readily detected, while the change in 5a and 5b is very great.

Vernon: How have you shown whether this compound has been oxidized or reduced? All you have shown is that it is present under some conditions and under other conditions it is gone.

Krall: I assume reductive conditions when oxygen is absent; also that light produces a reductant. 5a might be the oxidant, but it would be rather odd that it would pile up under reductive conditions. Of course, it might be a product of fermentation.

Rabinowitch: If this is the reduced product for the oxidation reduction system, wouldn't you under the conditions where it is oxidized find another peak, which would belong to the same compound in the oxidized form?

Krall: I feel that it goes into the 5b peak, but the evidence on that is tenuous.

Rabinowitch: Could 5a or 5b be anything related to thioctic acid? Has thioctic acid some characteristic absorption band?

Krall: The oxidized form has a 340-mμ band but I cannot find this band in 5a.

French: Have you tried any other plants?

Krall: Algae, corn, tobacco and barley. All contain both 5a and 5b.

French: In about the same concentrations?

Krall: The concentration in algae is not as high as in corn and barley.

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Phosphate in the Photosynthetic Cycle in *Chlorella**

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Studies on light-dependent phosphorylation in photosynthesizing organisms (*Chromatium*, strain D, and *Chlorella*) have been carried out in this laboratory since 1947 (1–3). With *Chromatium*, so far, only an introductory investigation was made; the more extensive part of the

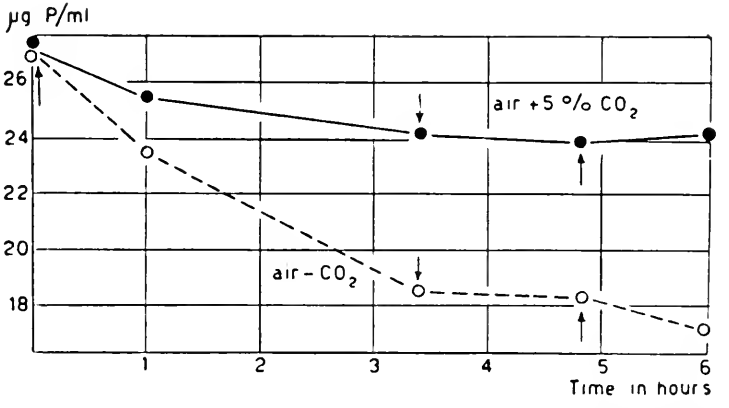

Fig. 1. Changes in the TCA-soluble phosphate of a suspension of *Chlorella* at pH 4.0, in light and darkness, and in the presence and absence of CO_2. ↑ Shift to light, ↓ shift to darkness. Expt. of May 30, 1950 (1b).

work has been carried out with *Chlorella*. The general trend of the phenomena is similar in both organisms (Fig. 1; *cf.* also refs. 1a and 1b). The following presentation deals especially with the *Chlorella* work.

It has been shown in experiments of long duration (several hours)

* A brief report along this line has also been given at the 3rd International Congress of Biochemistry at Brussels, 1955 (see *Résumés Communics.*, No. 11–16, p. 103).

† 132nd Communication of this Laboratory; 44th Comm. on Photosynthesis.

that light promotes the uptake of inorganic phosphate and its conversion into polyphosphates. Wintermans (3b) has shown that the compounds accumulating are probably polyphosphates; this conclusion was reached by the comparison of properties of these compounds with data in literature for polyphosphates in other organisms. A few of his observations will be listed here.

TABLE I. Decrease in TCA-soluble Phosphate in Suspensions of *Chlorella* after various Treatments. Summarized Results of Various Experiments (1c).

Treatment	PO_4 converted (in μg. P/ml.) in: 3 hr.	5 hr.	Number of expts.
1. Light; CO_2-free air	3.2 ± 0.7	5.0 ± 0.9	5
2. Light; CO_2-free air + glucose	1.7 ± 0.5	3.2 ± 0.5	5
3. Difference between rows 1 and 2	1.55 ± 0.3	1.8 ± 0.4	
4. Light; air + 5% CO_2	2.1 ± 0.5	3.1 ± 0.4	5
5. Light; air + 5% CO_2 + glucose	0.5 ± 0.5	1.9 ± 0.4	5
6. Difference between rows 4 and 5	1.6 ± 0.4	1.2 ± 0.3	
7. Dark; CO_2-free air	0.0 ± 0.4	0.4 ± 0.4	3
8. Dark; CO_2-free air + glucose	0.2 ± 0.4	0.0 ± 0.6	3
9. Dark; air + 5% CO_2	0.5 ± 0.1	0.7 ± 0.2	3
10. Dark; air + 5% CO_2 + glucose	1.0 ± 0.4	1.2 ± 0.4	3

The phosphate compounds accumulating are not soluble in cold TCA; they are easily extracted with hot 1*N* HCl, but undergo rapid hydrolysis; they are extractable by alkali with only slight hydrolysis. They move very little in a paper chromatogram (using solvents as described by Hanes and Isherwood, and by Bandurski and Axelrod, see 3b). They show metachromatic reaction with toluidine blue, and precipitation with Ba^{++} at low pH; the precipitate (in one case) was found to contain about 23% P.

The accumulation of polyphosphates continues for hours; its rate is increased in the absence of CO_2 (Table I). The rate becomes light-saturated in the absence of CO_2 at a much higher light intensity (viz., around 1.5×10^4 ergs/(cm.^{2} sec.)) than photosynthesis under similar conditions (around 3×10^3 ergs/(cm.^{2} sec.)). However, at a much lower light intensity than for photosynthesis (around 6×10^4 ergs/(cm.^{2} sec.)).

The accumulation of the phosphate compounds under consideration in the absence of CO_2 is maximal at pH $\sim$ 4, and decreases toward zero at pH 7 to 8. It does not require oxygen and is not affected by the presence of nitrate. It is less sensitive to phenylurethane than photosynthesis; only the photochemical reaction appears to be affected.

Photosynthesis and polyphosphate formation are affected by dinitrophenol in mutually similar ways. Also here the effect is greatest at low light intensities. pH has an effect upon this inhibition which can be understood quantitatively if one assumes that the cell membrane is impermeable to the ionic form of the poison, and, moreover, that

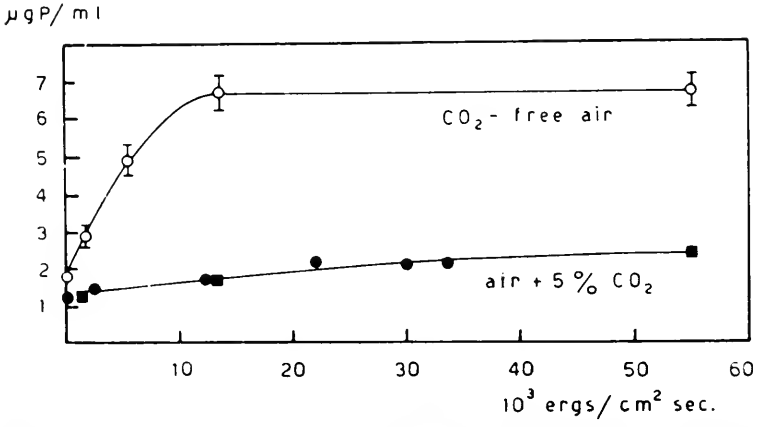

Fig. 2. Fixation of orthophosphate by *Chlorella* at different light intensities in the presence and in the absence of CO_2·pH ± 4.0; 25° C.; ± 5 mm.³ cells/ml.; illumination for about 3 hours (3b).

the internal pH of the cells is several units above 4 (3b). Sodium azide and sodium fluoride inhibit photosynthesis and polyphosphate formation in much the same way (3b).

Chromatium was found to excrete inorganic phosphate into the suspension medium in darkness (1a). *Chlorella* mostly fixes some phosphate, also in darkness. This, however, requires oxygen, contrary to the fixation in light. Under anaerobic conditions a release of phosphate may occur in darkness (3b); then the situation becomes very similar to that in *Chromatium* (1a). Dark fixation was found to be less sensitive to glucose, phenylurethane, and NaF, but more sensitive to DNP than fixation in the light.

We have investigated the time course of the conversion of phosphate, thus reproducing the well-known experiments of Kandler (4). We repeated these experiments because we wished to know the time constants of these phenomena for the cell material we used. The time

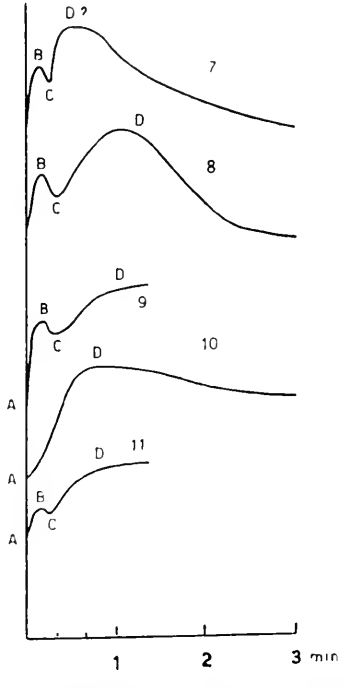

Fig. 3. Comparison of some induction phenomena in *Chlorella* photosynthesis. Ordinate: arbitrary units. Abscissa: time of illumination in minutes. Curve 7: Bioluminescence of *Chlorella*, after Strehler and Arnold (7). Curve 8: Same as curve 7 (8). Curve 9: Bound phosphate in nitrogen atmosphere (5). Curve 10: ATP-formation, after Strehler (8). Curve 11: Bound phosphate, in an atmosphere of air (5). (The whole figure from ref. 6.)

scale of the concentration changes of TCA-soluble phosphate in the cell material was abbreviated with respect to that of Kandler. The inverse curve was taken to represent the changes in TCA-insoluble phosphate formed (5). The time course and the sequence of maxima and minima showed a close affinity to maxima and minima of fluorescence and redox potentials (6). Moreover, a close affinity appeared

to exist between these curves and bioluminescence curves as reported by Strehler and Arnold (7, see Fig. 3), which suggests that the time constants of their cell material are much the same as in ours. On the other hand, the ATP time curve in Strehler's material (8) fails to show a transient maximum corresponding to the phosphate fixation maximum after 5 to 10 seconds, shown in our curves (see Fig. 3). This led us to the conclusion that this maximum represents a phosphate other than ATP. Its nature is now under investigation in our laboratory. In view of the fact that sulfhydryl-containing phosphates recently have been reported on (Krall, this symposium) and sulfhydryl compounds have been postulated to be closely connected with

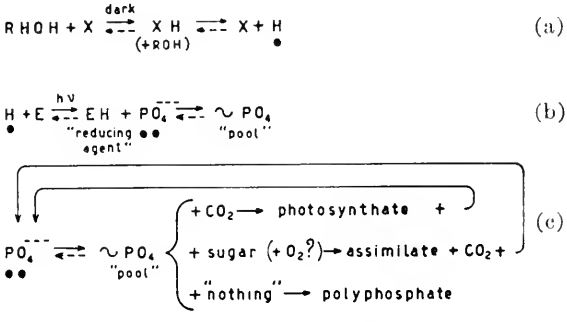

Fig. 4. Scheme, representing the relations between photosynthesis and polyphosphate formation. (Original.) Dots (● and ●●) indicate corresponding compounds in the subsequent lines of the scheme.

the energy transfer from chlorophyll (9), it will be of interest to inquire into the possible sulfhydryl content of the "10 sec." phosphate.

The way in which the light-dependent polyphosphate formation is linked with photosynthesis may be visualized as follows (see Fig. 4). The scheme also contains a suggestion concerning some reactions connected with the energy transfer. The first line (a) of Fig. 4 expresses the possible feeding in of hydrogen from the ultimate hydrogen donor (water) into the light-sensitive redox system E $\rightleftharpoons$ EH, acting as energy acceptor. The step E $\rightleftharpoons$ EH is considered to require light energy (or excited chlorophyll). The possibility of an intermediate step (X $\rightleftharpoons$ XH) is expressed. Possibly, EH combines with phosphate, giving rise to the 10-second phosphate, or one or two intermediate hydrogen transfers (EH + F $\rightarrow$ FH + G $\rightarrow$ GH, etc.) may

be intercalated (line b). During this period, the fluorescence curves are independent of CO_2 and insensitive to CO_2 reduction inhibitors (6,10). This suggests, in addition to other phenomena mentioned below, that phosphate is being taken up into the photosynthetic chain prior to CO_2. Probably within the time of about 1 minute (*cf.* Strehler's curve, Fig. 3) the EH (or F,GH) phosphate-compound formation gives rise to an (energy-rich) phosphate pool, probably ultimately filled mainly by ATP.

Line (c) contains the interpretation of the experimental results discussed above. The pool of organic, probably "energy-rich" phosphate is being continually refilled by the light reaction, according to lines (a) and (b). It is emptied, as far as our experiments go, along three different pathways: (i) in the presence of CO_2 the major part is used along the pathway of CO_2 reduction whereby it is reconverted into inorganic phosphate, suggesting a decrease of the overall conversion, which is measured; (ii) in the presence of sugar, an additional pathway (which probably may be denoted mainly as "oxidative assimilation") equally leads back to inorganic phosphate; (iii) when both (i) and (ii) are curtailed the formation of polyphosphate is observed, obviously resulting from "overfilling" of the organic phosphate pool.

The results underlying line (c) are strongly indicative of phosphate fixation in the photosynthetic chain in fairly immediate sequence of the light reaction, prior to the entrance of CO_2. Otherwise it could not well be explained how the absence of CO_2 might lead to increased polyphosphate formation. The underlying phosphate fixation as such, therefore, must be independent of CO_2 reduction and get light-dependent. The experimental results leading to this presentation were among the earliest demonstrations of a true "photosynthetic phosphorylation" (1).

Discussion

Krall: In answer to the question whether Dr. Wassink's bound phosphate might be the same as my compound 5a, I do not believe that to be the case. My 5a-5b complex is the last compound in the phosphate pool to become labeled with P^{32} while Wassink's bound phosphate is labeled easily.

Benson: Do you think that the polyphosphate reservoir might be an energy source?

Wassink: We, of course, have been aware of this question and we have investigated whether this reservoir can be emptied again. It appears that it can be

emptied but only very slowly. It is not a very readily available reservoir. It is comparable in some way to, e.g., starch, which also can be reversed but as a rule only very slowly. One thing that made me think it might be related to Dr. Krall's substance is his mentioning that it contains sulfur. We may ask whether it has anything to do with sulfur compounds which are now believed to be localized mainly in the beginning of the series.

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Photosynthetic Phosphorylation by Isolated Spinach Chloroplasts*

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In the light, but not in the dark, isolated chloroplasts esterified inorganic phosphate (P_i) into adenosine triphosphate (ATP). This light-dependent process has been termed "photosynthetic phosphorylation" (1). ATP accumulated (1,2) when illuminated chloroplasts were incubated under nitrogen with the proper cofactors and a phosphate acceptor, either adenylic acid (AMP) or adenosine diphosphate (ADP). The cofactors so far identified for photosynthetic phosphorylation by isolated chloroplasts are Mg^{++}, ascorbate, flavin mononucleotide (FMN), and vitamin K (3,4). The procedures for isolating chloroplasts are described in detail elsewhere (5).

The product of the reaction was identified as ATP (*a*) directly, by adsorption on Norite, followed by hydrolysis of the labile phosphate and (*b*) indirectly, using glucose plus hexokinase as an ATP-acceptor system, *either* during the process of photosynthetic phosphorylation *or* following the termination of the reaction and the chemical isolation of the ATP; in both cases the product of the hexokinase reaction, glucose-6-phosphate, was identified chromatographically (2).

The esterification of phosphate proceeded unimpaired under an atmosphere of purified nitrogen (3), showing that the presence of free oxygen is not a prerequisite for the occurrence of photosynthetic phosphorylation. In fact it was observed with whole chloroplasts that the presence of air actually depressed the phosphorylation when FMN and vitamin K were added as cofactors.

The process of photosynthetic phosphorylation has been represented (2,6) by the equations 1, 2, and 3.

$$h\nu + H_2O \xrightarrow{\text{chloroplasts}} 2[H] + [O] \qquad (1)$$

* Aided by grants from the National Institute of Health and the Office of Naval Research.

$$2[H] + [O] + n\cdot ADP + n\cdot P_i \xrightarrow{\text{chloroplasts}} H_2O + n\cdot ATP \quad (2)$$

$$\text{Sum: } h\nu + n\cdot ADP + n\cdot P_i \longrightarrow n\cdot ATP \quad (3)$$

in which [H] and [O] represent, respectively, the reduced and oxidized products of the photolysis of water by the chloroplasts. The

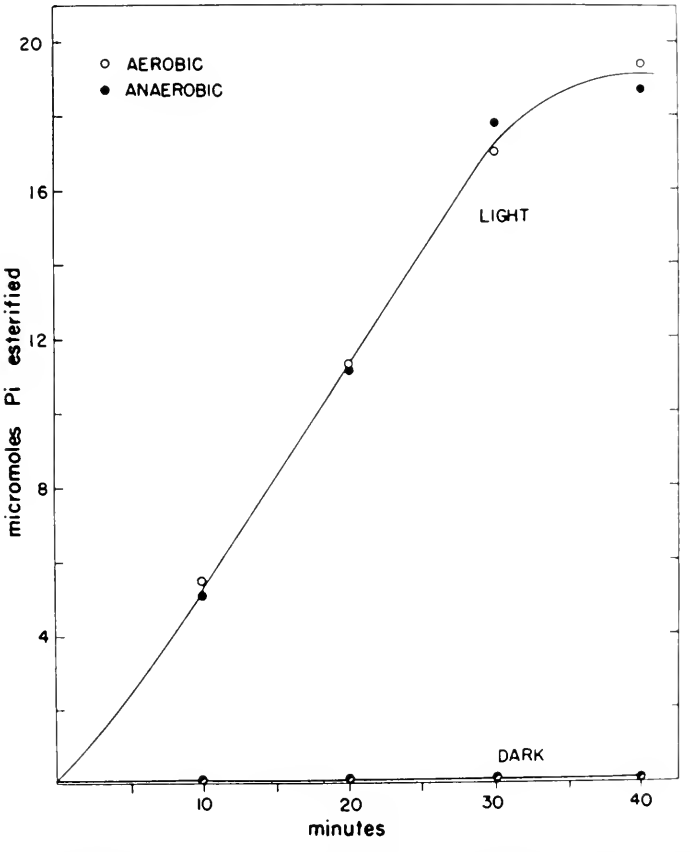


Fig. 1. Light dependence of photosynthetic phosphorylation by broken chloroplasts. The reaction mixture contained broken chloroplasts (chlorophyll content = 0.5 mg.), 40 μM tris (hydroxymethyl) amino methane, pH 7.4, 20 μM potassium phosphate, pH 7.2, 20 μM adenosine-5-phosphate, pH 7.4, 10 μM $MgCl_2$, 10 μM sodium ascorbate, 0.01 μM FMN, and 0.3 μM vitamin K_5, made to a total volume of 3 ml. with water. The reaction was carried out at 15°C. under nitrogen or air. Estimations of the phosphorylation were made as described previously (2,5).

symbol [O] is intended to express the experimental observation that phosphorylation proceeds in the absence of molecular oxygen (3,6).

Photosynthetic phosphorylation by whole chloroplasts was shown to be unaffected also by the presence or absence of carbon dioxide. If carbon dioxide was eliminated from the reaction mixture the phosphorylation proceeded unimpaired (2). It was also shown that whole chloroplasts fail to carry out any oxidative phosphorylation when supplied with Krebs cycle intermediates (5).

In earlier experiments active phosphorylation was obtained only with whole chloroplasts (1). It was found later that unbroken chloroplasts were not essential, provided that the proper cofactors were added to the reaction mixture (7). Washed whole chloroplasts were first isolated in 0.35 *M* NaCl and were then suspended in distilled water. By this treatment their gross structure was destroyed. Photosynthetic phosphorylation by the broken chloroplasts was found to be similar to phosphorylation by whole chloroplasts. The enzymes involved in photosynthetic phosphorylation are apparently unaffected by the water treatment.

The light dependence of the photosynthetic phosphorylation catalyzed by the broken chloroplasts is illustrated by the progress curves in Fig. 1. All of the inorganic phosphate added to the reaction mixture (20 μM) was used up in the light in 30 minutes, whereas virtually no esterification was observed in the dark. With the broken chloroplasts, the rates of phosphorylation commonly observed were twice those obtained with whole chloroplasts, suggesting that some permeability factor limits the rate with the latter.

The phosphorylation by the broken chloroplasts, like that by whole chloroplasts, proceeded unimpaired under anaerobic conditions, as shown in Fig. 1. This confirms the previous conclusion that free oxygen is not involved in photosynthetic phosphorylation.

The dependence of the broken chloroplasts on cofactors resembles that of whole chloroplasts. Table I shows the results of an experiment in which the known cofactors were added separately or in combination. Details of the optimal concentrations of the various cofactors will be presented elsewhere. All these cofactors are known to be components of green leaves (8), and some, like vitamin K, are characteristically concentrated in the chloroplasts (9).

It is likely that Mg^{++} has a catalytic role in the transfer of phosphate groups (10). The other cofactors may serve as electron carriers.

TABLE I. Flavin Mononucleotide (FMN), Ascorbate, Vitamin K, and Magnesium Ion as Cofactors of Photosynthetic Phosphorylation by Broken Chloroplasts

	Conditions	Micromoles P_i esterified in 30 min.
(A)	No cofactors added	0.07
(B)	10 μM Mg^{++} + 10 μM ascorbate	0.20
(C)	(B) + 0.01 μM FMN	8.3
(D)	(B) + 0.03 μM vitamin K_5	3.4
(E)	(B) + 0.01 μM FMN + 0.03 μM vitamin K_5	12.5[a]
(F)	(B) + 0.03 μM FMN	12.2
(G)	(B) + 0.3 μM vitamin K_5	13.9
(H)	(B) + 0.03 μM FMN + 0.3 μM vitamin K_5	14.2[a]
(I)	10 μM Mg^{++}, 10 μM ascorbate, 0.1 μM FMN, 0.03 μM vitamin K_5	20.0
(J)	(I), omitting Mg^{++}	5.9
(K)	(I), omitting ascorbate	2.9

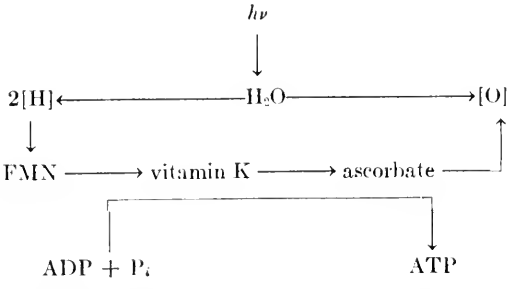
[a] Only at the low concentrations (arbitrarily chosen) of FMN and vitamin K_5 could an interaction be demonstrated. At the high concentrations no interaction was observed, indicating that the phosphorylation was then limited by some factor other than FMN or vitamin K_5.

The reaction mixture included, in addition to the above, 40 μM tris (hydroxymethyl) aminomethane, pH 7.4, 20 μM K phosphate, pH 7.4, 20 μM AMP, pH 7.4, and broken chloroplasts containing 0.5 mg. chlorophyll. The volume was made to 3 ml. with water. The reaction was carried out under nitrogen at 15°C. in an illuminated Warburg respirometer with continuous shaking. Phosphorylation was determined as previously described (2,5).

The amounts of FMN and vitamin K_5 needed indicate that they function as catalysts and not as substrates. Ascorbate, although supplied in larger amounts, is also used as a catalyst (3). The addition of pyridine nucleotides did not affect the phosphorylation by the broken chloroplasts, although it was found that most of the pyridine nucleotide contained in whole chloroplasts is leached out on treatment with water (7).

Broken chloroplasts, like whole chloroplasts, were unable to carry out oxidative phosphorylation in the dark with Krebs cycle intermediates or reduced pyridine nucleotides. This again indicates that the ATP synthesis by chloroplasts represents an anaerobic synthesis of pyrophosphate bonds at the expense of light energy by an enzyme system peculiar to photosynthesis.

On the basis of the evidence now available a tentative scheme for photosynthetic phosphorylation is presented below:



It is suggested that the light energy used for the photolysis of water is converted to the pyrophosphate bond energy of ATP during the transport of [H] to [O] via a series of electron carriers, of which three, FMN, vitamin K, and ascorbate, have so far been identified. The identity of the electron carriers beyond ascorbate is unknown, but they may very likely prove to be components of a cytochrome system (11–13). However, the independence of photosynthetic phosphorylation from molecular oxygen suggests that the typical cytochrome oxidase is not involved. The relative positions assigned to FMN and vitamin K in the "electron ladder" are tentative, and based solely on published redox potentials (14). It is possible that their positions are reversed *in vivo* (15).

Discussion

Rabinowitch: What kind of illumination did you use?

Whatley: Neon tubes containing a phosphor which gave predominantly red light but had a fairly wide spectrum.

Gibbs: Does ADP have any effect on the amount of phosphorylation?

Whatley: No, you get the same results whether you use ADP or AMP as acceptor.

Witt: What is the size of the broken chloroplasts?

Whatley: These range from approximately a micron up to the size of the unbroken chloroplasts.

Clendenning: To observe the full capacity for water photolysis in isolated chloroplasts, you have to use as dilute suspensions as when measuring the photosynthetic capacity of *Chlorella*. There should be less than 0.1 mg. chlorophyll per Warburg vessel. Most work on the Hill reaction refers to denser chloroplast suspensions. Hill reaction rates per unit of chloroplasts or chlorophyll are about 150% higher with 0.1 mg. than with 0.5 mg. chlorophyll per vessel. But I don't

think this would apply to your phosphorylation reaction. In comparing it with the Hill reaction, phosphorylation should be favored by high chloroplast concentrations.

Rabinowitch: You obtain from a preparation containing 0.5 μM chlorophyll about 12 μM ATP in a half hour?

Whatley: In a half hour you can get up to 20 μM fairly consistently.

Rabinowitch: Dr. Arnon suggested that it is unimportant—but I think it *is* important—to have a quantitative idea of the relative rates of the phosphorylation reaction and of photosynthesis. It seems to me that the first is only a few per cent of the second (assuming that the production of three high-energy phosphates is the equivalent of the reduction of one carbon dioxide molecule). You should not think that I am prejudiced against the phosphorylation reaction as something closely related to photosynthesis. I only suggest that it is important to know the relative rates.

Whatley: The maximum observed rate of phosphorylation is approximately 80 moles phosphate per mole chlorophyll per hour.*

Rabinowitch: This is about 40% of normal photosynthesis, on the basis of a single ATP per O_2 molecule.

Gaffron: I thought that oxygen production was inhibited by phthiocol and that, under the influence of this poison, phosphorylation replaced oxygen production. However, from the rates you report, it appears that these are not necessarily two competitive processes.

Whittingham: Chloroplasts can give 300 to 400 moles O_2 per mole chlorophyll per hour in the Hill reaction.

* Recently the maximum rate of photosynthetic phosphorylation which we have obtained was increased from 80 to approximately 400 moles phosphate per mole chlorophyll per hour.

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Part V

KINETICS, TRANSIENTS, AND INDUCTION PHENOMENA

On the Efficiency of Photosynthesis above and below Compensation of Respiration

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Kok has published figures which indicate that, below the compensation point, the efficiency of photosynthesis may be twice what it is above compensation. More recently, Bassham, Shibata, and Calvin (1) have reported similar results. It has been widely speculated (for example, Franck (2)) that, when respiration exceeds photosynthesis, the amount of energy required per molecule of photosynthetic oxygen production may be smaller than above compensation, because intermediates from oxidative metabolism may be serving as substitutes for carbon dioxide as the substrate for photosynthesis.

It is important to know whether photosynthetic oxygen production above and below the compensation point can represent different amounts of energy storage. We report here some comparisons of efficiency of photosynthesis above and below compensation.

The measurements were made with *Chlorella pyrenoidosa* cells suspended in carbonate buffer No. 9, saturated with 0.5% carbon dioxide in air. Pressure changes (representing oxygen exchange) were observed at 1-minute intervals by the method described by Emerson and Chalmers (3). A differential manometer and double cathetometer were used. Photosynthetic oxygen production was calculated from observations during alternating 10-minute periods of light and darkness. The calculated rates of photosynthesis are derived from the steady rates of pressure change observed during the second 5 minutes of each 10-minute period, but do not include the transition effects which were observed during the initial minutes of each period. The energy of the light beam was about 1.5 μ einsteins per minute at a wavelength of 644 mμ (cadmium line). Cell suspensions were dense enough for total absorption of this frequency (300 to 400 μl. cells in 8 ml. of liquid). Area of the light beam was about 4 sq. cm., and the area of the bottom of the reaction vessel was about 10 sq. cm.

Table I shows a comparison of the efficiency of two samples of cells,

whose respiration differed by a factor of about seven. In the case of the cells with low respiration, photosynthesis exceeded respiration. Whereas, in the case of the cells with high respiration, photosynthesis was less than respiration. The calculated efficiency was essentially the same in the two cases.

TABLE I. Comparison of Efficiency of Photosynthesis above and below Compensation of Respiration

		Cells with low respiration, 0.234 μl. O_2/hr. /μl. cells	Cells with high respiration, 1.72 μl. O_2/hr. /μl. cells
Pressure changes, mm.	5 min. dark, before illumination	−5.68	−54.90
	5 min. dark, after illumination	−5.67	−50.65
	Average per 5 min. dark	−5.67	−52.77
	5 min. during illumination	+6.44	−38.25
	Light action, per 5 min.	+12.11	+14.52
Calculation of efficiency	K_{O_2}	1.11	1.21
	μ moles O_2 per min.	0.120	0.157
	μ einsteins absorbed per min.	1.29	1.55
	ϕ^{-1}, quanta per oxygen	10.7	9.9

In these experiments, the light intensity decreased from the incident value and approached zero as the light penetrated the cell suspension. Thus the statement that photosynthesis was above compensation in one case and below in the other, means only that the *sum* of respiration for all cells exceeded or was less than the *sum* of photosynthesis. Certainly, the rate of photosynthesis in the full intensity of the incident beam must have been sufficient to produce photosynthesis above compensation, even in the case of the high respiration cells. This effect was counterbalanced by the cells exposed to lower intensities as the light penetrated the suspension and by the cells which were in darkness outside the periphery of the light beam, which was smaller than the area of the reaction vessel. The experiment therefore does not show whether there would be a difference in efficiency above and below compensation, under conditions of continuous illumination of all cells with approximately uniform light intensity.

In our opinion, comparison of quantum requirements of different samples of cells on the basis of measurements with thin suspensions

would be uncertain, because current methods of measuring the fraction of incident light absorbed by thin suspensions are not sufficiently precise. Methods of measuring scattered light are imperfect, and errors vary with distribution of scattered light, which in turn varies from one sample of cells to another. However, the question whether in thin suspensions the quantum requirement of photosynthesis is different above and below the compensation point may be

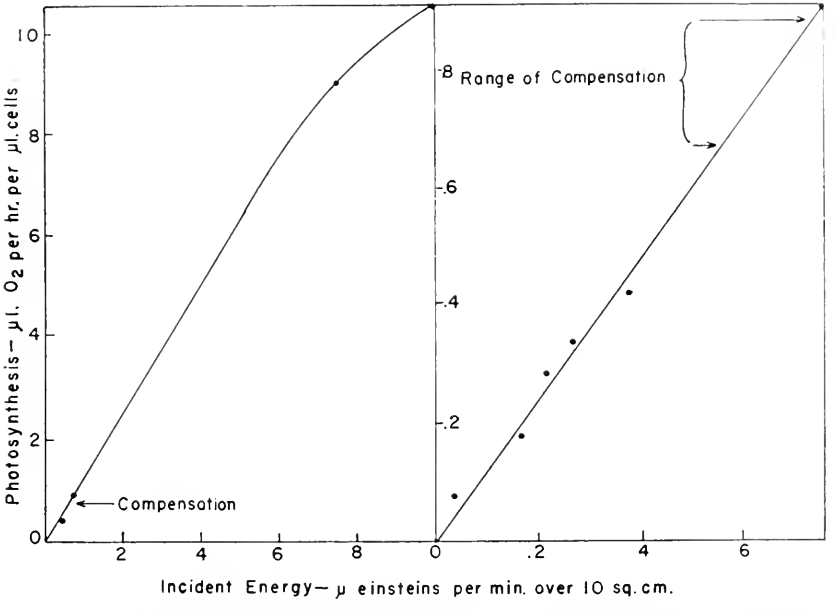

Fig. 1. Rate of photosynthesis as a function of light intensity, showing linearity through the region of compensation of respiration. The right-hand figure shows the lowest portion of the left-hand figure, plotted on an expanded scale so that all observations can be shown.

investigated simply by measuring the rate of photosynthesis as a function of intensity of incident light. We have made a number of such measurements with samples of cells grown and prepared in different ways. In all cases we have found that, within the limits of error of our experiments, the rate of photosynthesis is a linear function of light intensity from zero through the region of compensation of respiration and up to intensities several times compensation. We have never observed a change in slope at or near the compensation point.

Figure 1 shows the results of such an experiment. Here 30-μl. *Chlorella pyrenoidosa* cells were suspended in 8 ml. of carbonate buffer No. 9. Light intensity was varied by means of calibrated wire screens. The light source was a cadmium-mercury arc. On the left is shown the full range of intensities used. On the right, on a scale expanded approximately tenfold, are shown the points up to just above compensation of respiration. Since the rate of respiration varies with time and in response to the light intensity being used, we have indicated on the drawing the *range* of compensation in the course of the experiment, rather than a compensation *point*.

Other experiments have shown similar results. In all cases the rate of photosynthesis has been linear through the region of compensation. No combination of cultural and experimental conditions which we have yet tried has given any evidence of higher efficiency of photosynthesis below compensation.

Acknowledgment. This work was supported by National Science Foundation Grant G-1398.

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Report on Some Recent Results at Wageningen

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I. TRANSITORY RATES

B. KOK AND C. J. P. SPRUIT

In open manometers we could observe high initial pressure changes after illuminating or darkening *Chlorella* suspensions. The calculated rates, accepting *the photosynthetic quotient* $\gamma = -1$, corresponded to quantum numbers close to one.

But it appeared that in the transitory phases γ deviated largely from minus unity: The newly developed recording volumeter allows a wide variation of the ratio liquid phase to gas phase (V_f/V_g). High transitory rates were only observed in this apparatus (*cf.* Fig. 1) in case a small liquid phase was used (i.e., if both oxygen and carbon dioxide contributed appreciably to the volume changes). If the liquid phase is large ($V_f/V_g = 4$ to 6), CO_2 exchange contributes very little to the total volume change, even if acid suspension media are used. In the latter case the transitory effect is hardly noticeable and the volumetric time course is very similar to the one observed if the cells are suspended in buffer mixtures (which also obscure the influence of CO_2 on the readings).

In other experiments standard size vessels were used with the recording volumeter, additionally equipped with electrodes for measuring polarographic current and pH. This made it possible to make simultaneous records of changes in volume, pH (CO_2), and oxygen.

Under conditions of high O_2 and CO_2 concentrations, the response of oxygen exchange to light was instantaneous with the possible exception of a small induction. With sufficiently high CO_2 pressures a marked delay in the response of CO_2 exchange to a change in intensity occurs (e.g., during 30 seconds). In addition outbursts of CO_2 upon illumination are very often observed. This anomalous CO_2 exchange can quantitatively explain the high transitory rates, as observed with the manometric method.

Under conditions of low oxygen concentration we observed in sev-

eral instances a high initial rate of oxygen evolution upon illumination, which lasted a few seconds. Also a high initial uptake could be found upon darkening.

It appeared, however, that there was no correlation, between the transitory reactions of O_2 and CO_2. Variations of γ over a wide range

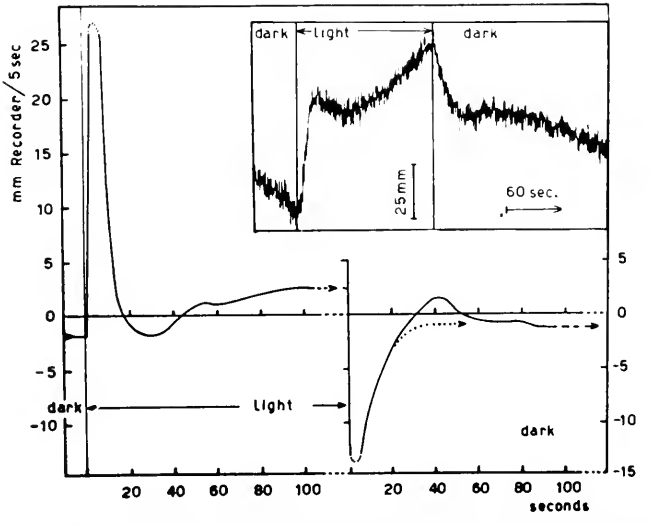

Fig. 1. Differentiation of a recording (reproduced in the inset) of transitory volumeter changes. *Chlorella* suspended in acid medium 10% CO_2 $V_f/V_g = 0.77$. After Kok and Spruit (1).

were observed. Without attempting to explain these complex phenomena, we concluded that polarographic experiments yield no evidence to support a one-quantum mechanism of the type proposed by Burk and Warburg (2).

II. KINETICS OF PHOTOSYNTHESIS

B. Kok and J. A. Businger

Extensive *flashing light* experiments were made with improved techniques of illumination and volumetry.

Flash saturation could still be obtained with flashes shorter than 10^{-4} second half-width, alternated with long dark periods. Above this limit the flash time could be adjusted to any value; the dark

period could also be varied within a wide range (from 10^{-3} second upwards).

Four types of experimentation were carried out for revealing the kinetics of photosynthesis:

1). Careful determinations of the intensity vs. assimilation curve in continuous light.

2). The "classical" flashing-light experiment: alternating brief flashes with dark periods of variable length. This was done for flash times ranging from 10^{-4} to 10^{-2} second.

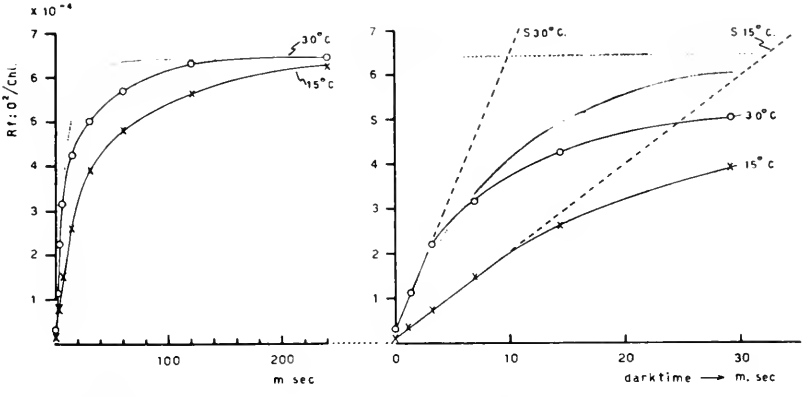

Fig. 2. Maximum flash yield as a function of dark time between flashes measured at two temperatures with *Chlorella* cells suspended in buffer mixture. In the right-hand drawing the time scale is expanded tenfold. Dashed slopes represent the saturation rate in continuous light. Dotted line indicates a first-order time course calculated for the observed maximum flash yield and initial slope at 30°C. Here $t_f = 0.5$ m sec. After Kok (3a).

3). Determinations of the intensity-rate curves in flashing light procedures similar to those followed in the experiments described in (*2*).

4). Flashing-light experiments, in which equal (long) dark times were alternated with flashes of variable length ($10^{-4}-1$ second).

Important plots are that of the maximum flash yield (R_{fm}) as a function of *dark time* (t_d) (measured with very brief, saturating flashes ($I \rightarrow \infty$, $t_f \rightarrow 0$)) (*cf.* Fig. 2) and that of the maximum flash yield (R_{fm}) as dependent of *flashtime* (t_f) measured with long dark periods ($I \rightarrow \infty$, $t_d \rightarrow \infty$) (cf. Fig. 3).

The R_{fm} vs. t_d plots appeared to be linear over a range of short dark times (e.g., in some cases at 30°C. beyond $t_d = 5$ m sec. and much longer at low temperatures) and were of complex character in the range of long dark times. Photosynthesis thus proceeds for a while at full saturation rate after the light is extinguished. This initial zero-order character of the reaction implies that the "half-times" as, e.g., observed by Emerson and Arnold (4) and confirmed by us, have no

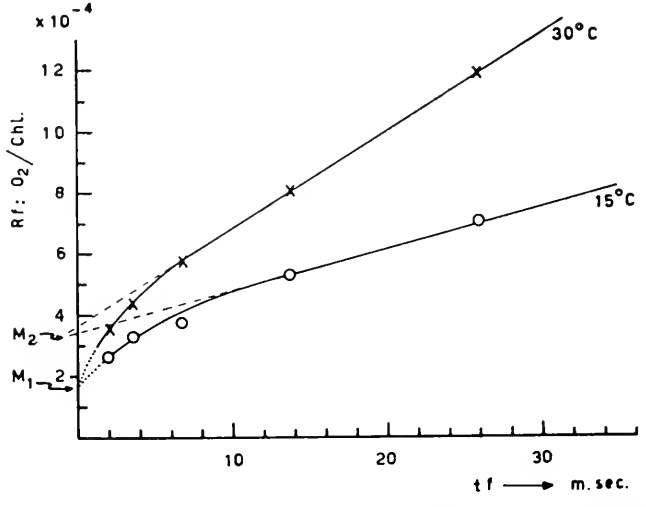

Fig. 3. Maximum flash yield as a function of flash time measured at two temperatures with *Chlorella* cells suspended in buffer mixture. Here $t_d = 0.1$ second. After Kok (3).

direct significance in terms of velocity constants. The maximum flash yield (U_0 for $t_f \rightarrow 0$, $t_d \rightarrow \infty$) is independent of temperature. The first conclusion to be drawn is that the primary photochemical product is a stable one. Intensity-rate curves, as measured in experiments mentioned under (*3*), were of an exponential shape, i.e., the primary photochemical product is formed in a one-quantum process. Thus in a single short flash one light quantum can be stabilized per 200 to 500 chlorophyll molecules ($U_0 = 2$ to 5×10^{-4} O_2/Chl), if we accept a quantum yield $\phi = 0.1$.

The R_{fm} vs. t_f plot (*cf.* (*4*)) yields a good deal more direct information:

The curve extrapolates to a finite flash yield for zero flash time (U_0). If the flash time is increased, the flash yield initially rises steeply according to an exponential time course. Further extension of the flashtime beyond 5 to 15 m sec. (dependent upon the algae used) yields a proportional increase of R_{fm} with flash time. This final slope is equal to the saturation rate in continuous light. The described type of results can be most simply interpreted by the mechanism:

(1) $U \xrightarrow{k_1 I} U^*$ $\qquad (U) + (U^*) = (U_0) = 2 \cdot 10^{-4}\ O_2\ Chl^{-1}$

(2) $U^* + E \xrightarrow{k_2} U + E^*$ $\qquad (E) + (E^*) = (E_0) = 6 \cdot 10^{-4}\ O_2\ Chl^{-1}$

(3) $E^* \xrightarrow{k_3} \phi(O_2) + E$ $\qquad k_2\ U_0 = 370\ sec.^{-1}$, $k_3 = 92\ sec.^{-1}$

U represents a photochemical "unit" or a "holochrome," a complex of (a few hundred) cooperating pigment molecules with a common acceptor. The absorption of a single light quantum within the unit suffices for its excitation; further absorption acts are fruitless until restoration via a subsequent dark reaction (2) has occurred. Another dark step (3), which may be located anywhere in the chain of reactions between light and oxygen, limits the restoration of E.

The four types of measurement discussed above yield more than sufficient information for evaluating the concentrations and time constants involved. The numbers given with the reaction sequence (1)–(3) (derived from an experiment made at 30°C.) may serve as an example. Relatively large variations may occur, depending upon the algae and their pretreatment.

It must be made clear that only with certain types of algae did the above mechanism suffice for a quantitative and accurate description of the observations. The more general formulation of the rate-limiting photosynthetic reactions probably is more involved.

III. PHOTOINHIBITION

B. Kok and J. A. Businger

Reaction vessels of a volume as small as 40 μl. are currently used in conjunction with our recording volumeter. Full-scale span of a Brown recorder then corresponds to about 0.05 μl. of oxygen. The area to be irradiated amounts to only 15 mm.^{2} and extremely high light intensities can be concentrated onto the algae (up to 1000-fold higher than required for photosynthetic saturation).

Under a variety of conditions we exposed algae to strong light and followed the time course of the decay of the rate.

Only with certain types of algae could results as described by Myers and Burr (5) be obtained, namely, an apparent saturation at

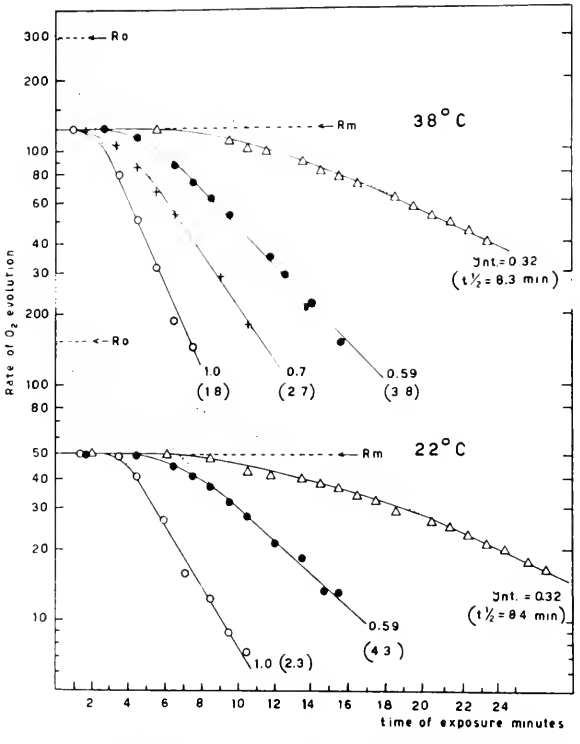

Fig. 4. Progressive decay of the photosynthetic (saturation) rate in strong light of various indicated intensities and at two temperatures. Half-times of the slopes are indicated in parentheses. After Kok (6).

intermediate photosynthetic rates over a range of medium light intensities before the onset of photoinhibition. Such cells show a relatively fast (temperature-dependent) dark-recovery reaction. The final level represents an equilibrium between photochemical destruction and dark restoration. Other algae do not clearly show recovery reactions during or after the decay. In most cases the photosynthetic saturation rate does not decay immediately upon illumination, but

only after a certain time has elapsed (*cf.* Fig. 4). This decay appeared to have first-order character and was only slightly influenced by temperature. These experiments suggest that a component that is usually present in excess under conditions of light saturation is photochemically destroyed. The rate in weak light (the quantum yield), on the other hand, decays immediately upon addition of too strong light.

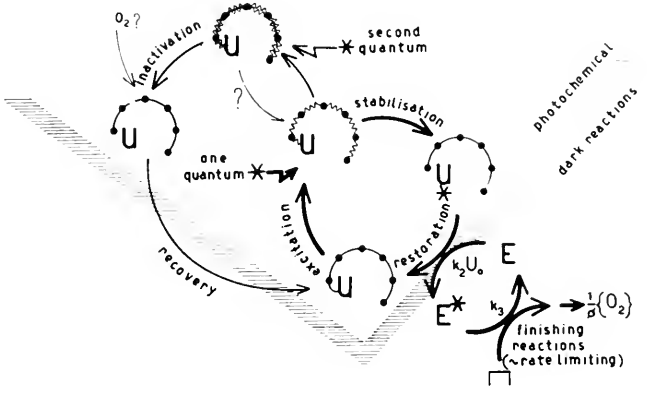

Fig. 5. Light and dark reactions in photosynthesis (bold arrows) and photoinhibition (thin arrows, left side). A complex of pigment molecules with its stabilization center U is excited by absorption of a single quantum and may be inactivated by a double absorption act. In a fast dark reaction (k_2U_0) the photosynthetically activated complex is restored by enzyme E. A slower dark reaction (k_3) in turn limits the restoration of E.

The maximum flash yield (R_{fm} for $t_f \to 0$, $t_d \to \infty$) also decays and does so in close parallelism with the decay of the quantum yield. From these observations we concluded that photoinhibition involves a photochemical inactivation of the pigment system and more specifically the inactivation of U or of the link between U and its group of pigment molecules (*cf.* our chapter on flashing light).

Further kinetic studies in continuous and flashing light indicated that a two-quanta process is involved and that photosynthetically excited units (U* in the preceding chapter) are only slightly (or not) susceptible to this photoinactivation; i.e., photoinhibition may result from the coincidence of two light quanta in a non-excited photosynthetic pigment complex. The action spectrum of photoinhibition

appeared to differ from that of photosynthesis; blue light is relatively more active in the former.

Though it is still a subject of study, we are inclined to accept the view that photoinhibition is only indirectly correlated with photo-oxidation. In the latter process the oxygen consumption might possibly be sensitized via pigment molecules which are disorganized in the former. Figure 5 summarizes our kinetic model of photosynthesis and photoinhibition.

Discussion

Rabinowitch: When speaking of the primary photoproduct, what do you mean by "stable?" Compared to what? To the Emerson-Arnold period of 0.02 second?

Arnon: What is the time between flashes?

Strehler: How do you explain the slower than linear increase of flash yield with the longer dark time?

Kok: In each flash, all the primary acceptor, U, is converted into U* (*cf.* sequence 1–3) and this component, in turn, rather quickly converts E into E*. The slowest step is the formation of oxygen with regeneration of E. So far, it has been impossible accurately to measure the time course of the oxygen evolution after a single flash. Instead, we apply a regular sequence of flashes; under such conditions, a steady state is approached in which, during the flash, as much U* is formed as is removed in the subsequent dark period.

During a relatively short dark time, only a fraction of U* is drained off. After the first few light-dark cycles, all the available E will be converted into E* while E molecules liberated by reaction (3) are needed to react with U*. Due to this "buffer" action, the rate of oxygen evolution will be constant during not-too-long dark periods; as long as this is true, the flash yield will be proportional to the dark time, and the slope of the yield vs. dark time curve will be equal to the maximum rate in continuous light.

The range of dark times over which this linear relation holds depends on the type of algae; at 30°C. it may extend up to 5 or 10 m sec. When the dark time is extended so much that a substantial fraction of U* is used up during it, the regenerated E molecules will not find a reaction partner. Reaction (3) will then slow down and the rate of oxygen evolution (which is equal to $k_3(E^*)$) will drop. In this picture the component U* is supposed to be "stable," i.e., to have a lifetime long enough to permit its "reloading" with E molecules for some time after the flash. A minimum demand for this lifetime would be of the order of 30 m sec. at 30°C., and longer at lower temperatures.

Rabinowitch: The difference between what you are now suggesting and what was more or less generally assumed in the past, is that the dependence of the flash yield on the length of the dark period is (within certain limits) linear and not exponentially declining, because the first photoproduct can be stored for a while, and the enzyme can operate on it several times—not just once, as in Franck's theory.

Tamiya: What is the order of the dark reaction in your model?

Kok: We cannot assign a definite order to the flash yield vs. dark time curve. This is so because each measured point is obtained under a steady-state condition of intermittent illumination. One has to postulate a certain reaction sequence, set up and solve the corresponding equations, and compare the solutions with the experimental findings.

Witt: Is the dark time dependence the same for the different flash times?

Kok: We have measured flash yield vs. flash time curves with dark times between 0.02 and 2 seconds. The curves measured with very long dark times (sufficient for all dark steps to run to completion) are easiest to interpret, but experimentally they are more difficult to obtain with sufficient accuracy.

Rabinowitch: In connection with Kok's mechanism, I wonder how common in enzyme kinetics is the assumption that a substrate can be transferred from one enzyme to another by a bimolecular reaction?

Gaffron: Kok presented it so nicely one would like to believe it is that simple. But there are some questions one may ask. For example, it appears that one can grow algae with "units" of different size. Is there a difference in the chlorophyll content? It is much easier to think that you can vary the number of enzyme molecules than that you can vary the size of chlorophyll units.

Rieke in our laboratory once showed that there are two different dark periods, one which is sensitive to cyanide, and another one—the Blackman-Emerson period—which is not. I mention this as an example of the fact that we have to reckon with a succession of enzymes, each of which might become limiting under different conditions.

Kok: Both the concentrations of U and E may vary, as expressed in relation to chlorophyll concentration. Rieke's cyanide-sensitive enzyme may be identical with our E.

Rabinowitch: I don't think that the explanation of Rieke's results by Franck (suggesting that a second dark reaction—a cyanide-sensitive and temperature-dependent carboxylation—can become rate-limiting instead of the Emerson-Arnold reaction) is sufficient to explain all the results.

For example, according to Gilmour, you can get the same effect of long dark intervals also in the Hill reaction, in which carbon dioxide is not involved, and which is not cyanide-sensitive.

A second difficulty is that, in cells used in Tamiya's experiments, the maximum steady rate of photosynthesis does not seem to have been significantly lower than in Emerson and Arnold's algae. Therefore, one cannot assume that, in Tamiya's case, another enzyme was, for some reason, deficient, and depressed the yield below the limit fixed by the Emerson-Arnold enzyme. Rather, one would have to make the unlikely assumption that the usual enzymatic "ceiling" was replaced by another one having about the same height as the usual one. I do not deny that the carboxylating enzyme can use longer dark periods—as Franck suggested—but its effect must come on top of the complications we are now discussing.

Gaffron: On the matter of photoinhibition: at very low oxygen tensions, you should not be bothered by photooxidation. Furthermore, photooxidation may be proportional to the square of the light intensity. If, for example, photooxidation is due to a peroxide, it is very likely that the formation of the latter will require two

elementary light reactions. The rate of each will be proportional to light intensity. In the final step, two OH-groups will have to combine at a rate proportional to the square of their concentrations. I think one could get Kok's quadratic term in this way.

Kok: Photooxidation and photoinhibition are not the same thing. As to the mechanism which results in a "two-quanta process," I am open-minded; but probably it has to occur within a single pigment unit.

Wassink: We decided formerly upon a kind of photosynthetic unit, similar to Wohl's "kinetic model" (*Enzymologia, 10,* 365–372 (1942)). I think this theory is consistent with what Kok found, or at least it can be made consistent with his finding. It simply implies that a quantum, or whatever it is, cannot travel through the whole amount of chlorophyll present, but only over a certain fraction of it, which may consist of, let's say, 400 or 600 chlorophyll molecules—or whatever number you may derive from the experiment. In this average area "it" is captured by an acceptor molecule (U). As soon as you knock out, say, four out of five, or two out of five, of the acceptor molecules (U), this mechanism does not work any more, or works less adequately. I think that is a connecting link between your scheme and Rabinowitch's kinetic suggestion.

Kok: The first intermediate, in which the excitation energy, absorbed by chlorophyll, is stabilized can be conceived either as a "floating" enzyme, or as a "reservoir" structurally and functionally tied up with its chlorophyll molecules in some kind of a "unit."

The observation that this "reservoir" is loaded by a first-order process in respect to light intensity indicates that a one-quantum process is involved. Therefore, rather than to accept that all the 4, 8, or 10 quanta, ultimately required for the evolution of one oxygen molecule, are successively stored in one and the same primary stabilizing molecule, it is simpler to assume—and more likely—that each center stores and transfers the energy of one quantum at a time. This, then, decreases the required size of the "unit" by a factor of 4, 8, or 10—say, from about 2000 to approximately 400 chlorophyll molecules per stabilization center.

In the alternative concept, the stabilization centers can be conceived as enzyme molecules floating about at random between, say, 400 times more numerous, unorganized pigment molecules. Then, in order to retain a high efficiency of light utilization in weak light, we have to ascribe a sufficient lifetime to the excited chlorophyll complex, long enough to allow for the diffusion of the enzyme to it (or the photoproduct must live long enough to diffuse to the enzyme and react there).

In the absence of functional or structural correlation between C and U (i.e., if virtually each chlorophyll molecule could react with each U molecule available), a decrease in the number of U molecules (which can be computed from the maximum flash yield) would only very gradually impair the efficiency in weak light. As long as excitations occur sufficiently infrequently, a few U molecules would suffice for the stabilization of all photoproducts. On the other hand, since U is involved in a rate-limiting dark reaction (as flashing light data show), one would expect the saturation rate to be far more strongly dependent upon the concentration of U.

Photoinhibition now provides a means for decreasing (U): the maximum flash

yield decays immediately upon exposure to excessive light. However, it is found that the quantum efficiency also decays immediately (but the saturation rate only after some delay).

This indicates that after the inactivation of a molecule U, a large number of C molecules are deprived of their outlet for excitation energy; this is our main argument in favor of assuming a functional cooperation between U and an assembly of C's—at least, of a regular spatial distribution of the two components.

But even with the "statistical" interpretation of the "unit," we still have a choice between two possibilities.

(*a*) The primary photoproduct (C*) could diffuse, as a chemical entity, to U (or conversely U could diffuse to C*). Then we have to accept that the lifetime t of C* is long enough to allow the enzyme U to "scan" 400 pigment molecules. In this model, the size of a "unit" could be determined solely by the value of t and the diffusion velocity of U. But we can repeat here the arguments used above: If t were much longer than the time required by U to reach C*, a decrease of (U) by photoinhibition would only gradually become manifest as a decrease in maximum quantum yield (since U molecules in this model are able to invade each other's areas). If t did not, or did only barely, fulfill the requirements set by the diffusion velocity of U, severe light losses and low quantum yields would be the consequence.

To prevent such losses by accepting a safe (sufficiently long) value for t, we have to return to the concept of a restricted "working area" assigned to each individual U molecule.

(*b*) The other alternative is energy migration through a series of pigment molecules by way of inductive resonance. Such migration has been shown actually to occur extensively in the chloroplast. This "physical" diffusion of excitation energy toward a final trap yields a satisfactory interpretation of the unit. Our relatively simple kinetic scheme satisfies most observations made so far; the assumptions of a relatively long lifetime of C* would require an additional reservoir between light and U.

Rabinowitch: I have pointed out repeatedly in the past that all experiments in which the same inhibition (for example, by ultraviolet light, or by excess light, as found by Kok, or by narcotics) is observed in strong and in weak light, require a correlation between saturation rate and maximum quantum yield. If, for example, you have a surface covered with chlorophyll, a molecule of the limiting enzyme must be assigned to a certain surface area and permitted to serve only the molecules of chlorophyll contained in this area. Thus, knocking out a molecule of the enzyme will "inactivate" all chlorophyll molecules in the whole service area.

I am not against a "physical" unit; I am only saying that the only reason the unit theory fits the kinetic results is because it permits the energy quantum, absorbed in one point, to end up at another point where it is needed, e.g., because this is where an enzyme molecule is available. However, any chemical product, formed at the original place of absorption, can similarly diffuse to the enzyme that has to service it (or this enzyme itself can go around and gather up the product). All that is needed is a division of spheres of influence between the individual chemical "messengers," or enzyme molecules, each being assigned exclusively, or at least preferentially, to a certain group of pigment molecules. I don't see

how at the present time, one could make a choice between the alternatives—energy diffusion or particle diffusion. The abundant experimental confirmation of energy migration in the chloroplast between different pigments—to which Kok refers—requires only a single energy transfer during the excitation time and cannot be taken as proof that the migration between identical molecules actually extends over several hundreds of the latter.

Strehler: If the absorption changes that Dr. Witt mentioned yesterday (a rapid change, followed by a slow fall-off) are due to the same compound, then,

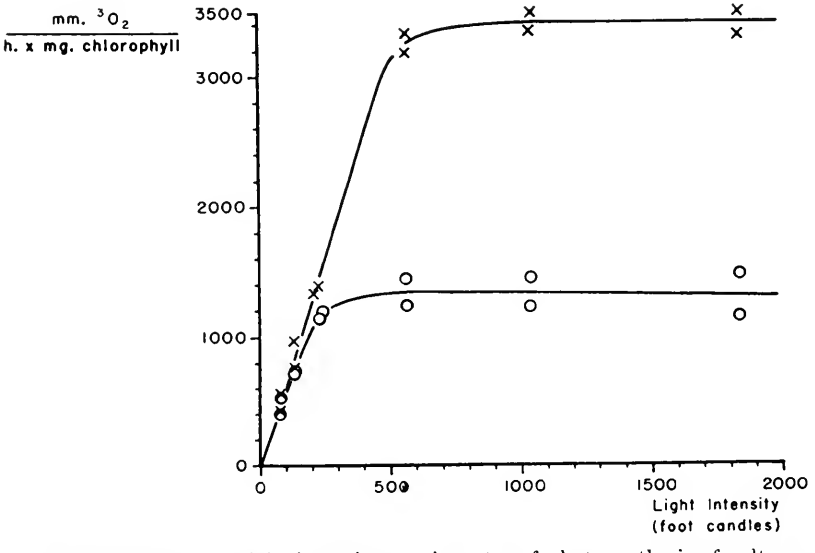

Fig. 6. The effect of light intensity on the rates of photosynthesis of cultures of *Scenedesmus* D-3 grown on a complete, highly purified mineral medium with an added 10 parts per billion of vanadium (×), and of cultures grown on the same medium without added vanadium (○).[7] Cells suspended in 0.1*M* $KHCO_3$-K_2CO_3 buffer pH 9.0 for measurement of photosynthetic rates. Rates of O_2 production corrected for dark respiratory O_2 uptake.

rather than saying, as Kok did, that there are two separate catalytic components, one reacting with another, it may be that there is a single component, which is adsorbed at the site of the photochemical process. This compound could dissociate after the photochemical act and be replaced by another molecule just like it. Diffusion would perhaps be limiting for the second process.

Kok: A model of this type, though apt to be more complicated, may also fit the observations.

Strehler: With respect to Rabinowitch's interpretation of Kok's scheme: It may not be necessary to assume that enzymes are involved at all in some of these steps, because it is known that some of the very important intermediate hydrogen

donors, such as riboflavin and naphthaquinone, can easily be reduced and oxidized in the absence of any apoenzymes. Therefore, it may be that this is simply a transfer of hydrogen from one reducing agent to another, without the intervention of a specific catalyst.

Lumry: In so far as I can determine from a rather casual look at Kok's data, there is a marked qualitative similarity between his results and those obtained by Gilmour from flashing-light studies of chloroplast fragments. Quantitative differences seem explainable if one assumes that the fragments had about one-fourth of the algae's concentration of the enzyme limiting the slow dark step. This is the factor relating the oxygen-liberation rates at light saturation in the two systems.

We, too, have observed an inhibition by excess light, but only at relatively high pH values.

Frenkel: Gaffron earlier mentioned that cells can be grown with "units" of different size, and asked about their chlorophyll content. The relation should be reflected in the photosynthetic saturation rate in continuous light, expressed on chlorophyll basis. We have some data on *Scenedesmus*, showing the effect of growth conditions on saturation rate which are independent of chlorophyll content. The experiment shown in Fig. 6 was performed in Dr. Arnon's laboratory. Cells were cultured in the presence and absence of vanadium; those grown in vanadium-deficient medium for 2 or 3 days showed a marked depression of their light saturated rates. At low intensity little or no difference was apparent. Thus, vanadium deficiency appears to affect primarily one (or several) dark reactions. It would be of interest to get flashing-light data of the kind Rieke and Gaffron had obtained (with grouped flashes), in order to localize further the action of vanadium.

Bassham: There seems to be a real deficiency in these algae; the chlorophyll content is lower.

Arnon: Under conditions of vanadium deficiency, *Scenedesmus* shows a growth deficiency, while *Chlorella* does not; yet both show an effect of the lack of vanadium on photosynthesis. (Compare Warburg's recent work on *Chlorella*.)

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Mechanism of the Initial Steps in Photosynthesis*

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Many experiments have been carried out with intermittent light in the hope of elucidating the mechanism of the initial steps of reactions in photosynthesis. But most previous studies have been concerned with the change of the photosynthetic yield per flash of a fixed duration with different dark periods. From the results, various theories were proposed, including the theory of a "photosynthetic unit" (1,2). Tamiya and Chiba (3) found that the maximum yield becomes temperature-dependent when the light intensity is very high. In place of the "photosynthetic unit" theory, they proposed a different mechanism in which the sensitizer S (probably chlorophyll) is converted to the excited sensitizer (S*), after which S* reacts with other chemicals to bring about photosynthesis or is deactivated by a second-order temperature-dependent reaction. Despite differences in these theories, there is similarity in one respect in that they assume only one compound to be excited or one reservoir to be filled during the course of a flash, with other steps in photosynthesis occurring during a subsequent dark period.

The preliminary experiment reported here was carried out to study the effect of the flashing-light period on the maximum yield per flash when a long dark period and a sufficiently high intensity are provided to saturate photosynthesis even with the flashing light. As will be shown later, it appears that at least two reservoirs are filled, or partially filled, in the course of light periods used in these experiments.

METHODS

The system used in this experiment to measure photosynthetic rate was the same as the system for our quantum requirement experiments (4), although the total volume was changed to increase the sensitivity, and the surface area of the cell containing the algal sus-

* The work described in this paper was sponsored by the U. S. Atomic Energy Commission.

pension was reduced to obtain more light per unit area of the suspension of algae. The photosynthetic rate was measured by observing the change of oxygen content in the closed system by means of an analyzer measuring the paramagnetic property of oxygen. At the same time, the change of CO_2 was observed as a reference by an infrared CO_2 analyzer. To saturate the photosynthetic yield, two 1-kw. projection lamps and a 400-watt photospot illuminated the suspension of algae through three sheets of infrared absorbing glass with a water-cooling system. In front of the cell, which contained 25 ml. of

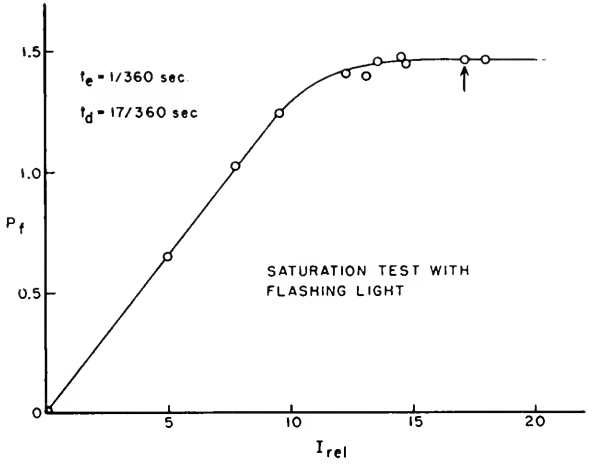

Fig. 1. Saturation test with flashing light.

algal suspension, a sectored disc was rotated. Six kinds of discs were used; they have 1, 2, 3, 4, 6, and 9 open sectors of the same size in a disc which is 40 cm. in diameter. The size of opening was made so as to obtain equal dark and light periods with a 9-hole disk. Therefore, dark periods relative to the light period in these discs are 17, 8, 5, 3.5, 2, and 1, respectively. If the rotating rate is fixed, the light period (t_e) is the same for all discs, but the dark periods (t_d) can be changed by changing the disc. From a set of data with a single rotation rate, one can obtain the change of yield per flash as a function of dark period for a fixed light period. The light period was changed by changing the rotation rate. The light periods were $^1/_{360}$, $^1/_{180}$, $^1/_{90}$, and $^1/_{45}$ second.

An important requirement in this experiment is that the photo-

synthetic rate be saturated with respect to light intensity under the conditions used. The saturation was tested with the longest dark periods (one-hole disc) for two light periods, $^1/_{360}$ second and $^1/_{45}$ second. One of the results, where $t_e = {}^1/_{360}$ second and $t_d = {}^{17}/_{360}$ second, is shown in Fig. 1, where relative photosynthetic rate per flash (P_f) is plotted against the relative light intensity (I_{rel}). The arrow in the figure indicates the light intensity at which all other experiments reported here were carried out. With this light intensity, the yield is well saturated even with the one-hole disc. The concentration of the suspension of *Scenedesmus* was 0.2% in packed cell volume units.

RESULTS

The results are shown in Fig. 2, where the relative photosynthetic rate per flash (P_f) is plotted against the dark period (t_d). Each curve

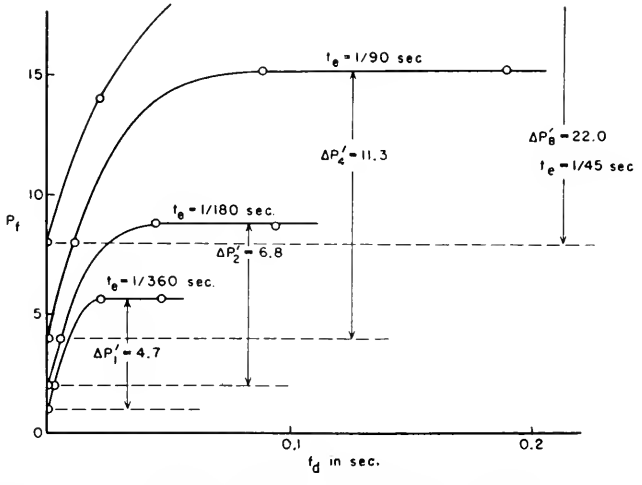

Fig. 2. Change of oxygen generation per flash with changing dark periods.

represents the change of photosynthetic rate for a particular light period (t_e) as a function of the dark period. The points for zero dark period were calculated from the continuous light rate. The relative unit of P_f was chosen so as to make P_f equal to unity for $t_e = {}^1/_{360}$ second and $t_d = 0$ second; hence the values for the intercepts for the curves at $t_e = {}^1/_{360}$, $^1/_{180}$, $^1/_{90}$, and $^1/_{45}$ second are 1, 2, 4, and 8, re-

spectively. As can be seen in this figure, P_f increases linearly at short dark times and levels off to a maximum value. The behavior of this change of P_f as a function of the dark period agrees with the results which have been obtained by other investigators.

For the purpose of discussion the effect of the variation in light period, $\Delta P'_e$ was defined as the difference between the P_f value for zero dark period and the maximum P_f value for each curve. The values of $\Delta P'_e$ and the corresponding absolute values of ΔP_e are listed in Table I.

TABLE I. Extra Yield of Oxygen per Flash in Photosynthesis as a Function of Flash Time

Ch (total chlorophyll-*a* content) = 117×10^{-8} mole/25 ml. of suspension

t_e	$\Delta P'_e$	ΔP_e (10^{-8} mole of O_2/25 ml.)	ΔP_e/Ch (%)
1/360	4.69	3.80	0.325
1/180	6.75	5.62	0.480
1/90	11.23	9.12	0.782
1/45	22.00	17.80	1.517
0 (extrapolated)	3.00	2.43	0.204

$\Delta P'_e$ is the measure of oxygen generation per flash in the dark in excess of the oxygen generation in the light. We postulate that, in the course of light period, a first reservoir is filled by the energy of light and must be full by the end of the light period because the photosynthetic rate is saturated against light intensity. Presumably, this initial step of photosynthesis would be a photochemical reaction, and some purely chemical reaction may follow it with the additional possibility of some deactivation process. But, if only one reservoir is filled during the light flash and is responsible for all further reactions of photosynthesis, $\Delta P'_e$ should be constant and independent of the light period, because the light intensity is strong enough to saturate the reservoir in any light period used. This would be true, no matter what the mechanism of reactions relating to this particular reservoir might be.

In Fig. 3, $\Delta P'_e$ is plotted against relative light period. As can be seen, $\Delta P'_e$ increases with increasing light period instead of remaining constant—the anticipated "saturation" is indicated by the dotted horizontal line. The rate of increase becomes almost linear at longer light periods. This result indicates that there is a second reservoir which is

partially filled even in these rather short light periods. The second reservoir begins to be filled almost from the beginning, as can be seen from the fact that the straight part of the curve can be extrapolated to pass through the origin. The ratio of ΔP_e (absolute value) to total chlorophyll content of the sample of algae is listed in Table I. The

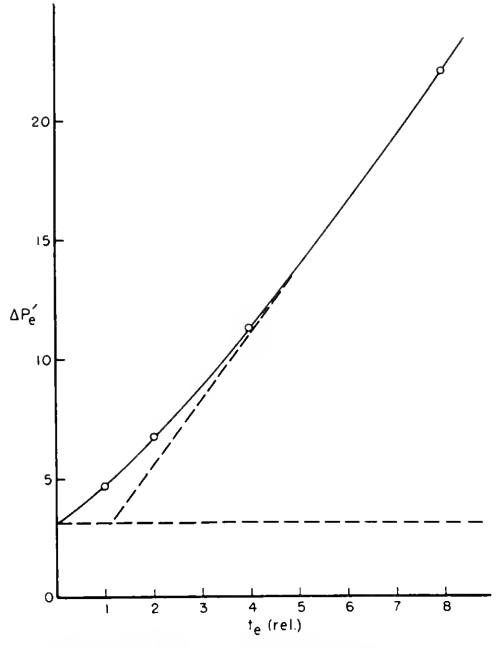

Fig. 3. Oxygen generation per flash with change of light period.

ratio extrapolated to zero light period is 0.204%, the reciprocal—about 481—is the number of molecules of chlorophyll for each molecule of oxygen evolved. This number is much smaller than the previously reported photosynthetic unit of 2500. It may be that the value of 2500 resulted from weak light intensities employed in earlier experiments.

Another fact we might point out is that the first reservoir is almost filled at about 3 msec. This time is calculated from the intersection of the horizontal straight line and the extrapolated straight part of the curve in Fig. 3. This time corresponds to the first fast decay period of chemiluminescence, which was observed by Strehler *et al.* (5).

The straightness of the curve in Fig. 3 at longer light periods indicates that the second reservoir is filled by a zero-order reaction, which is quite reasonable for an enzymatic reaction. If the light period is further increased, the curve in Fig. 3 should level off when the second reservoir is saturated.

Discussion

Tamiya: How high was your maximum yield per flash?

Bassham: The values are given in Table I, but it should be mentioned that some algae give five times higher yields per flash than others depending on growth conditions.

Tamiya: What is the factor determining the variation?

Bassham: The yield depends principally upon the chlorophyll content of the algae. When one prolongs the flash time, one obtains two or three times higher oxygen yields than with a short flash. This effect increases as the temperature increases.

Tamiya: Are you sure that your shortest flashes were saturating?

Bassham: Yes.

Tamiya: The dark period for saturation was much longer than that in Emerson's experiment?

Bassham: Yes, much longer.

Whittingham: We did some work on flashing light; it was dropped because of the War. One difficulty that bothered us then was that, when one shakes a *Chlorella* suspension, one superimposes upon the flashes given from the outside flashes owing to the cells' being moved from a region of relative high light intensity to one of relatively low intensity and back.

I wonder whether Dr. Bassham or Dr. Kok could tell us what was the percentage absorption of incident light in their suspensions.

Shibata: Less than 50%. Even if variations in intensity caused by shaking were as high as 20%, this did not matter because the light was far above saturation.

Rabinowitch: The present difficulty with the theories based on the original experiments of Emerson and Arnold is that Tamiya has since discovered, and Kok confirmed, a yield-enhancing effect of dark periods much longer than the original "Emerson-Arnold period" of about 10^{-2} second at room temperature, which becomes apparent when the flashes last longer than 10^{-4} second, and that in this case the flash yield becomes temperature-dependent. This indicates that there is some kind of a "reservoir" of intermediates, which longer flashes can fill and from which material for further transformation can be extracted in prolonged dark periods.

Gilmour, who has obtained similar results in the study of the Hill reaction, devised a rather complicated mechanism. In his theory, there is a side reservoir, in which the primary photochemical products are stored, later to be taken back into the main reaction sequence. Kok finds that a simpler mechanism is sufficient. All one needs, according to him, is to substitute a two-step transformation of the photoproduct for a one-step transformation; one of the two enzymatic components (Kok's E) acts as a "reservoir," filled up by longer lasting flashes.

In the original interpretation of the Emerson-Arnold short-flash experiments, two constants had been derived. One was a *concentration*, $5 \cdot 10^{-4} \times n$(Chl); it was supposed to be either the concentration of "photosynthetic units," or that of a "finishing" enzyme (enzyme B in the terminology of Franck and Hertzfeld), n being the number of times the enzyme has to act for one molecule of oxygen to be liberated.

On the other hand, from the duration of the dark period needed to saturate the flash yield, a *rate constant* (50 second^{-1} at room temperature) was derived, which was interpreted as the action constant of the same enzyme. We note that these two constants were derived quite independently—one from the maximal flash yield, the other from the length of the dark period needed to attain this maximum. If Kok's mechanism is substituted, the first constant retains its significance as the concentration of "units" (U), but the second "constant" is replaced by a function of the rate constants of the two reaction steps. Therefore, the decay function ceases to be representable by a simple exponential curve. However, with *brief* flashes, the second of Kok's two steps dominates the kinetics, and the decay is approximately exponential, with a rate constant (*ca.* 0.01 second) similar to that derived from Emerson and Arnold's experiments. However, the concentration constant and the rate constant now apply to different catalytic components. The picture has thus become less simple than before, but it seems to be able to account for results obtained with both long and short flashes.

I do not see, however, the amended theory as providing new arguments for the existence of photosynthetic units as physical entities. I believe Kok could rewrite his equations in terms of two successive independent enzymes, E_1 and E_2, instead of a "unit," U, and an enzyme, E. Instead of postulating a unit which is excited as a whole—a unit containing $2000/n$ chlorophyll molecules—one can assume that one molecule of an enzyme is present per $2000/n$ molecules of chlorophyll, and photoproducts formed by all of the latter must be worked up by the one enzyme molecule. The only new element in Kok's picture is that, instead of enzyme E_1 transforming the primary photoproduct directly into the final product, it now hands it over—after some sort of change—to a second enzyme, E_2, which transforms it into the final product.

Whittingham: That is exactly the way Briggs had formulated the problem.

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Chemical-Kinetic Studies of the Hill Reaction*

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INTRODUCTION

We have continued the line of investigation reported at the first Gatlinburg conference in an attempt to secure a higher-plant chloroplast system suitable for precise and definitive measurements of chemical kinetics of the Hill reaction. Most biological systems, including those able to carry out photosynthesis, are usually so difficult to control as to make kinetic studies with them a rather hit-or-miss affair. Initial experiments suggested that chloroplast fragments from higher plants might prove simple enough to allow control at the level found necessary for a fundamental understanding of the chemistry of simpler systems and now affording such rapid progress in protein and enzyme investigation. To judge from the history of chemistry, precise kinetic investigations will have to be coupled with extensive biochemistry if we are ever to understand the mechanism of photosynthesis at more than a superficial level.

It cannot yet be said with absolute confidence that chloroplast fragments will provide an ideal system but our efforts to date support our early optimism. We would like to report briefly the progress in control studies as well as some observations about the kinetic patterns which are developing.

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EXPERIMENTAL DETAILS

Both the manometric and potentiometric techniques were used for determining Hill reaction velocities as previously described (1). The potentiometric technique was preferred because of its greater precision, speed of measurement, and applicability at low electron acceptor (oxidant) concentration, as will be discussed later. Most of the experiments were carried out with washed sugar beet, Swiss chard, or rhubarb chard chloroplast fragments prepared and stored as previously described (2). Chloroplast fragments stored in 0.5 *M* sucrose at −35°C. did not show any loss of activity or change in other properties for periods of at least one year. If stored samples of the same batch of material were removed at intervals and thawed according to a precise time schedule under conditions of controlled temperature, it was found over extended periods that Hill reaction velocities were obtained which agreed within two or three standard deviations of the method (1.5% to 3%) used for rate measurements. At the present time it is of importance to do all the experiments in a series with samples from the same batch of chloroplasts, since it has not been found possible to prepare different batches of material with identical properties. One or more preparative variables are not yet under control. Plant growth conditions also have a marked effect on chloroplast properties. For example, it has been suggested by preliminary experiments in this laboratory that chloroplasts from sugar-beet plants grown under short photoperiods show a large decrease in the Hill reaction rate per chlorophyll molecule at light saturation. It may be that natural inhibitors for one or more of the subprocesses of the Hill reaction are formed under certain environmental conditions and that these inhibitors cannot be removed by the usual washing techniques. Alternatively, and more probable, necessary cellular participants in the Hill reaction may be partially removed or chemically altered (as by oxidation) during the preparatory process, and they may be reduced in concentration as a result of particular combinations of growth conditions.

Chloroplasts prepared under nitrogen from plants (especially sugar beet) grown under mid-summer conditions exhibit up to a 50% increase in Hill reaction activity after being incubated for a short time at 20° to 25°C. (3). Values of $Q_{O_2}^{Chl}$ of over 3000 have been obtained with sugar-beet chloroplasts prepared in this manner with ferricyanide as the oxidant. This activation phenomenon is confined to the rates

obtained at saturating light intensities. The high velocities obtained with such material may result from the removal of natural inhibitors or from the prevention of inhibitor formation during the activating process. In this sense these high rates may represent the true intrinsic chloroplast reaction. On the other hand, since activation is in general obtained only with chloroplasts prepared under nitrogen, it may be that some oxygen-sensitive, high-energy compound is present which can contribute a part of the energy requirement of the Hill reaction. Treatment with oxygen or other oxidants during illumination removes the activation effect.

The most serious problem encountered in attempting to make precision rate measurements is the rapid second-order inactivation reaction previously described (3). Two additional observations by Bishop on this spontaneous reaction are worth noting: (*1*) Both low-light and high-light slow steps are decreased in exactly the same way; (*2*) the reaction requires oxygen, as judged from the observation that of a variety of attempts made to slow down the loss rate, only a very considerable reduction of the oxygen partial pressure had a large effect.

THE RELATION BETWEEN LIGHT INTENSITY AND HILL REACTION RATE

It is necessary in this chemical-kinetic approach first to establish the relation between reaction rate and light intensity. Then any proposed mechanism for the process, to be correct, must first of all satisfy the requirements of the observed rate vs. light intensity kinetic relationship. A tremendous number of light curves, especially for photosynthesis, have appeared in the literature. Early work in this laboratory resulted in light curves which closely approximated rectangular hyperbolas. However, the variability was such that it was not possible to say that the relationship was actually that simple. With improved techniques Rieske (4) in this laboratory has shown that the relation is actually that of a rectangular hyperbola to a high level of significance. He has been able to establish by statistical methods that the following steady-state rate law (which is in the form of a rectangular hyperbola) provides an excellent fit to rate data secured with finite layers of reaction mixtures (up to several millimeters) with optical densities up to 0.60:

$$V_I = \frac{kI_A}{K + I_A} \tag{1}$$

where V_I is the experimentally determined velocity of the Hill reaction resulting from the light intensity averaged through the reaction cell, I_A; k and K are constants. If the true rate law obtaining throughout an infinitesimal depth of reaction system (microscopic law) were:

$$v = \frac{k_L k_D I}{k_L I + k_D} \tag{2}$$

where v is velocity through an infinitesimal reaction cell depth along the axis of propagation of the light beam, k_L is the rate constant for the limiting light step, k_D is the composite rate constant at infinite light intensity, and I is the constant intensity within the reaction layer, we would have expected a better fit to the experimental data from the following equation which averages velocity through a cell of finite depth

$$\bar{V} = \frac{kI_0 [1 - e^{-\alpha a}]/\alpha a}{K + I_0 [1 - e^{-\alpha a}]/\alpha a} \tag{3}$$

K and k are constants, I_0 is the incident intensity, a is cell thickness, and α is the absorption coefficient of the suspension. The fact that the use of average light intensity provides a better rate law than the use of average velocity indicates the presence of a complication previously suggested, but now of greater importance in view of certain newly established ramifications of flashing-light kinetics (see B. Kok's paper in this volume). The observed behavior in the Hill reaction as described above is consistent with the interpretation that there is a time lag so great between photon absorption and product formation that the chloroplast fragments can pass through regions of very different light intensity during the lag. Equation 2 would thus be expected to yield the rectangular hyperbola of equation 1. In such a situation one really observes flashing-light effects dependent on the rate of stirring in supposedly steady-state experiments. We have always found rapid stirring necessary to produce maximum rates of reaction at high light intensities. Simple calculations show that the decrease in intensity of the light beam in passage through a single chloroplast fragment or an algal cell would be sufficient to produce flashing-light effects even if the chloroplast fragment or algal cell merely rotated in place. Only experiments at the very high light intensities sufficient to produce saturation throughout the chloroplast fragment or the cell would thus give the true steady-state picture.

However, by the use of equation 2 we can, and routinely do, extrapolate through some inverted linear form of this equation to zero and infinite light intensities at which conditions all three of the equations give identical results. The two rate parameters k_L and k_D can

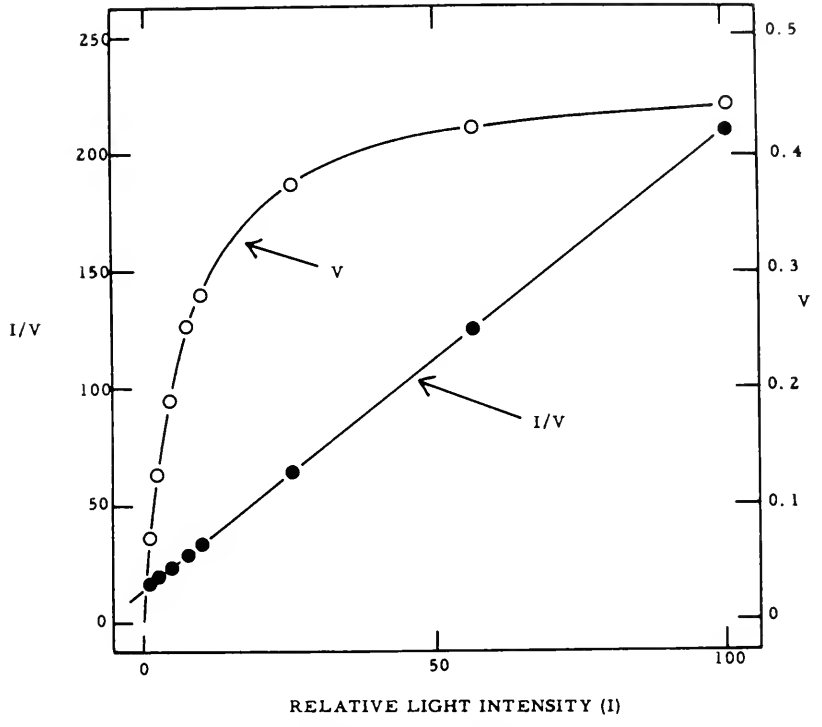

Fig. 1. Curves showing the rectangular hyperbolic nature of the Hill reaction-light intensity relationship for isolated chloroplasts. Each point represents the mean of eight separate rate measurements. Here I is the relative incident light intensity in arbitrary units, while V is the rate expressed in terms of moles ferricyanide reduced per mole chlorophyll per minute. The initial ferricyanide concentration was 0.00025M, the pH was 6.80, and the temperature was 2.5°C.

thus be determined by least-squares extrapolation. This permits the calculation of the true steady-state rate at any intensity, providing the true microscopic rate law is given by equation 2. A set of data obtained under controlled conditions is shown in Fig. 1. The data given are the means of eight sets of measurements and are plotted

both in the form of a rectangular hyperbola (V vs. I) and in a linear form (I/V vs. I), where V is the Hill reaction rate and I is the relative light intensity. In order to illustrate the precision which can be obtained, the slopes and intercepts as calculated by the method of least squares for the eight sets of data in Fig. 1 are given in Table I. It will also be noted that equation 2 sets very stringent requirements upon any kinetic mechanism proposed to explain the Hill reaction. These restrictions will be discussed in another paper.

TABLE I. Slopes ($1/k_D$) and Intercepts ($1/k_L$) Defined as Given in Equation 2 and Calculated by the Method of Least Squares from the Same Data Which Are Plotted as Means in Fig. 1. Each Set of Data Consisted of Rate Measurements at Eight Different Light Intensities

Data Set	Slope	Intercept
1	2.15	14.0
2	2.13	14.4
3	2.13	14.7
4	2.13	14.0
5	2.12	14.8
6	2.08	14.9
7	2.14	14.9
8	2.08	15.1
Mean	2.12	14.6
Standard deviation	0.01	0.4
Per cent	0.5	2.9

EFFECTS OF HILL OXIDANTS ON THE HILL REACTION

A wide variety of compounds will serve as electron acceptors for the Hill reaction of isolated chloroplasts. Considerable work has been carried out in attempts to define precisely the kinetics of the Hill reaction in relation to oxidant properties and reaction conditions. Mehler and Brown (5) have shown that dissolved oxygen, a product of the Hill reaction, will also act as an oxidant. Oxygen at ordinary partial pressures cannot compete for electrons to a measurable extent in the presence of the usual oxidants such as ferricyanide and benzoquinone. It can, however, compete with a few substances such as chromate and cause an apparent decrease in the rate and an apparent alteration of the stoichiometry of the Hill reaction. On the other hand, oxygen, even in trace amounts, may complicate experiments through its participation in a rapid back-oxidation of Hill oxidants after they are

reduced. The rapidity of this effect, which appears to be catalyzed by fragments, varies widely with different oxidants. Within experimental error, rates as measured with ferricyanide, for example, are independent of oxygen partial pressure up to 1 atm. except for lengthy runs at low light intensities. Some substituted naphthoquinones, on the other hand, are so sensitive to back oxidation that they can be used only under highly anaerobic conditions. This effect is probably responsible for the various reports on the relation between the half-cell potentials of different oxidants and Hill reaction velocities rather than any fundamental relationship between reducibility of oxidants and the reducing power of illuminated chloroplasts. The literature shows clearly that under proper conditions illuminated chloroplasts can reduce very "poor" oxidants such as TPN about as rapidly as "good" oxidants like ferricyanide and benzoquinone (9). It can be suggested, then, that most Hill oxidants act in about the same way and permit about the same maximum rate of reaction when artifacts are eliminated. As a consequence the energy requirements for photosynthesis and the Hill reaction must be about the same. In this laboratory quantum requirements for the Hill reaction with light of 6750 A (as extrapolated to zero light intensity) of 8 have been obtained—a result which is in good agreement with the values for photosynthesis as obtained by Emerson, Daniels, and many others.

The Hill reaction rate of chloroplast fragments is linearly dependent on chloroplast concentration (as measured by chlorophyll content) over a rather wide range. When studied as a function of oxidant concentration, however, it appears that there are three distinct regions of behavior as the oxidant concentration varies from 10^{-7} M to 10^{-1} M. The Hill reaction is very difficult to study at extremely low oxidant concentrations (10^{-7} M to 5×10^{-6} M) because of back-oxidations due to oxygen and because even highly washed chloroplast fragments retain some reducing power which cannot be conveniently oxidized away without altering the chloroplast properties. In this low oxidant region the rate of the Hill reaction drops off rapidly with decreasing oxidant concentration. It is probable that in this range the reaction rate is limited by oxidant concentration according to a first-order diffusion process.

A middle region with respect to the relation between oxidant concentration and Hill reaction rate is found in the range 5×10^{-6} M to 10^{-3} M. As shown with ferricyanide as oxidant in Fig. 2, the reac-

tion is essentially independent of oxidant concentration over this range with rate nearly identical for many different oxidants, although this fact has not yet been unequivocally established. Since the overall reaction is not limited by oxidant concentration in this range, it would appear that the free energy of activation of the oxidant reduction process is probably quite low if not zero. This strongly

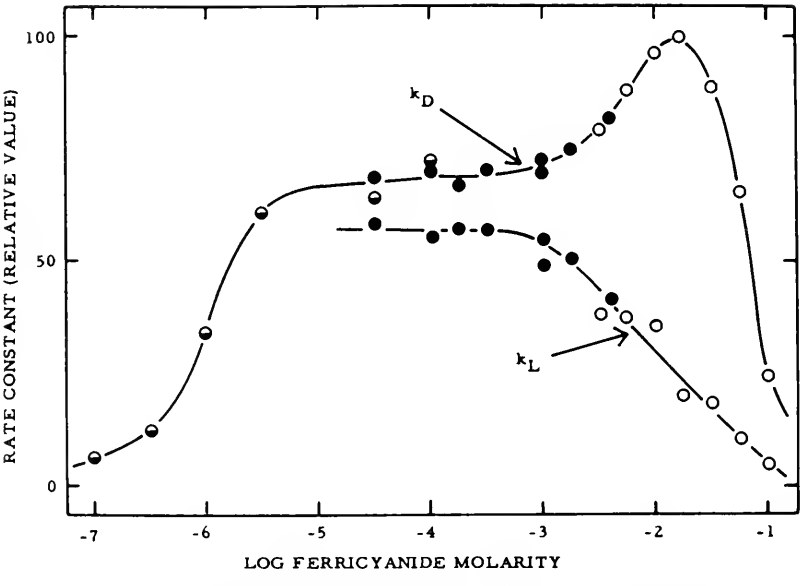

Fig. 2. Curves showing the relation between oxidant concentration and the relative values of the light-reaction-rate parameter k_L and the dark-reaction-rate parameter k_D for the Hill reaction of isolated chloroplasts as defined by Equation 2. Open circles, manometric method; closed circles, potentiometric method; half-solid circles, manganese dioxide method.

suggests that the oxidant is reduced by a very high-energy intermediate in the overall reaction—a fact consistent with the low specificity requirements for oxidants.

The middle region demonstrates the simplest kinetics. It is thus the most useful range for studying the Hill reaction, since we will probably find here the basic mechanism of the natural reaction least confused by artifacts.

When oxidant concentration is progressively increased above

5×10^{-4} to 10^{-3} M several complicating effects appear. As shown in some detail for quinone in Fig. 3, the rate of the limiting light reaction (k_L) decreases rapidly and approaches zero as the oxidant concentration exceeds 10^{-2} M. The rate of the limiting dark reaction (k_D), on the other hand, rises to a maximum at a concentration of slightly less than 10^{-2} M, after which it decreases rapidly. This same general

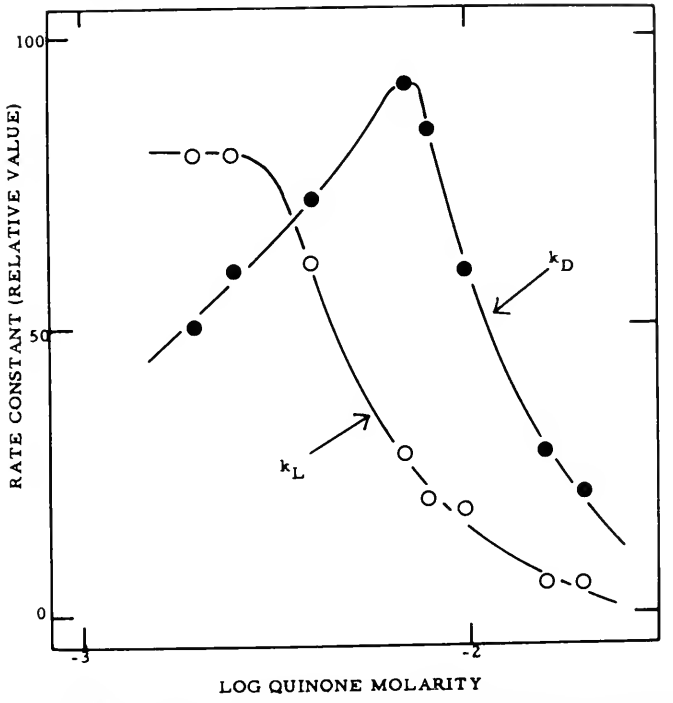

Fig. 3. Curves showing the relation between oxidant concentration and the relative values of the light-reaction-rate parameter k_L and the dark-reaction-rate parameter k_D for the Hill reaction of isolated chloroplasts as defined by equation 2. The reaction was measured manometrically with benzoquinone as the oxidant.

behavior was shown by all the oxidants studied, though there are distinct quantitative differences. Studies in this concentration range are also complicated by the so-called chloride effect (6). We have found in this laboratory that the relative activating effect of oxidant concentration on k_D is dependent on the chloride ion concentration of the reaction system. These complications may be of some importance in

studies of the overall photosynthetic process (for example, see the remarks which follow the paper of A. H. Brown in this volume.) However, they represent a serious problem in kinetic studies of the Hill reaction, since they occur in the range of oxidant concentration customarily used in manometric measurements of Hill reaction activity. Bradley and Calvin (7) in studies on the participation of thioctic acid found effects of this compound which appear to be closely related to those described above. The quenching of chloroplast fluorescence with increasing oxidant concentration, as studied in this laboratory by Mayne, follows a relationship rather similar to that demonstrated by k_L as described above. It is apparent that oxidants exert complicated effects on chloroplast properties in this high concentration range. Thus, all studies of the Hill reaction made under such conditions must be examined with great care in order to distinguish between the fundamental processes involved and secondary reactions which may be artifacts. In order to avoid these problems, most of the work discussed in this paper was carried out in the middle oxidant range, where complications seem to be at a minimum. If this could not be done, at least some studies were made to establish that effects were the same in both the middle and the upper concentration ranges.

Slightly soluble oxidants cannot be studied with the usual manometric methods, since the acceptor must be at least several thousandths molar in order that enough oxygen will be evolved to be measured conveniently. A new technique has been developed in this laboratory in which an excess of an inert, insoluble terminal electron acceptor is added to the chloroplast system together with a low concentration of the electron acceptor being studied. As the experimental electron acceptor is reduced by the illuminated chloroplasts, it will in turn be converted back to the oxidized form by the terminal acceptor. This technique was used by Hochster and Quastel (8) in studies on a number of respiratory systems. Such an approach also permits measurements to be made at a constant redox potential and in the very low concentration ranges of soluble oxidants.

It was found that colloidal manganese dioxide would reoxidize at a useful rate a number of compounds which were of interest as possible electron acceptors for the Hill reaction and which could not be studied by the usual manometric techniques. Slightly soluble compounds such as toluquinone, anthraquinones, riboflavin, *o*-benzo-

quinone, dichloroquinone, phthiocol, 1-methyl naphthoquinone, and other vitamin K analogs, etc., were good acceptors, while ferricyanide could be studied at concentrations as low as 10^{-7} M. A number of compounds of theoretical interest, such as 6-thioctic acid, 5-thioctic acid, and glutathione, did not function as direct electron acceptors in our experiments.

HYDROGEN ION DEPENDENCE OF THE HILL REACTION

Robin Hill, in his original work, pointed out the great dependence of the reaction rate on the pH of the suspending medium. Photo-

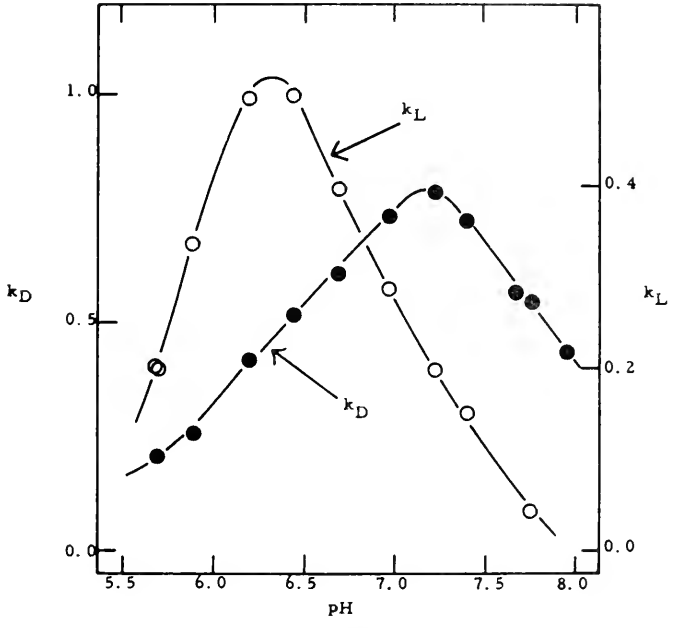

Fig. 4. Curves showing the relation between pH and the relative values of k_L and k_D for the Hill reaction of isolated chloroplasts as defined by equation 2. The reaction was run at eight light intensities and the reaction rates were determined by the potentiometric method (2). The reaction was carried out in 0.1M potassium phosphate buffer.

synthesis in living cells is much less sensitive to the hydrogen-ion concentration of the supporting fluid, probably because of the presence of pH-regulating mechanisms. It is likely that detailed studies

of the relation between pH and the Hill reaction rate of isolated chloroplasts will ultimately provide the kind of fundamental information now beginning to appear in the field of enzyme mechanism. The most detailed study of the pH dependence of the Hill reaction is the recent work of Rieske (4), who determined the pH dependence of k_L and k_D as shown in Fig. 4. The effect of k_L closely approximates the relation between pH and the quantum yield of isolated chloroplasts, as reported by Wayrynen *et al.* (9) in that both show a maximum at approximately pH 6.3. This result was to be expected since the quantum yield is proportional to k_L.

A similar maximum is shown by k_D but at much higher pH. Above 7.5 the Hill reaction becomes very sensitive to small changes in suspending media and preparative conditions and in particular to traces of inorganic ions in a manner not yet understood. For this reason the true optimum of k_D may lie at a higher pH than thus far found. There is a further complication resulting from the occasional appearance of an inhibition by light. Just how much parallelism will ultimately appear between these pH studies and similar studies on algae and intact cells is, of course, not yet known. However, if much similarity does exist those growth conditions and reaction components upon which the internal cell pH depends will have to be much more carefully controlled than in present practice. For example, a shift in internal pH of 0.5 unit could result in a two fold change in quantum yield (see Fig. 4).

EFFECTS OF DEUTERIUM OXIDE ON THE HILL REACTION

Most studies under steady-state illumination and in flashing light of the effects of deuterium oxide on photosynthesis have yielded relatively simple results. The only effect has appeared to be a decrease in the rate of the limiting dark reaction (10). Although these studies have not received much theoretical consideration they might be interpreted in terms of water entering the overall photosynthetic process at the Blackman step, since the changes in apparent free energy of activation observed are comparable in magnitude to those found in a simpler chemical system where activated-complex formation involves the breaking of bonds containing hydrogen. Horwitz (11) and Krasnovskii and Brin (12) studied the effects of deuterium oxide on the Hill reaction of whole *Chlorella* cells and on leaf-chloroplast preparations, respectively.

In this laboratory Rieske (4) has studied the effect of deuterium oxide on the chloroplast Hill reaction as a function of pH and of light intensity. The results are much more involved than those reported for photosynthesis in that both the light and the dark parameters are

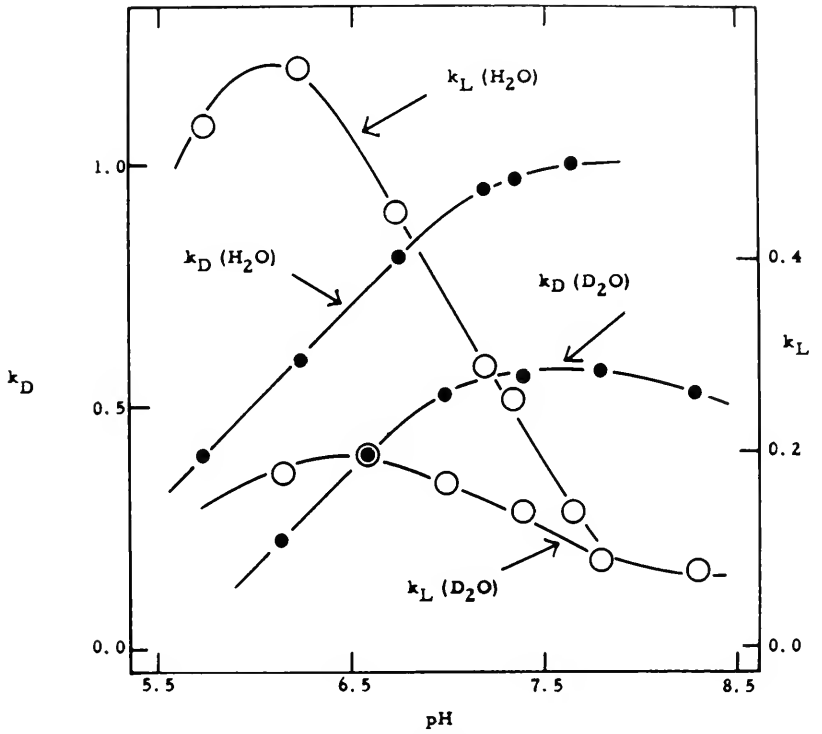

Fig. 5. Curves showing the values of the rate parameters k_L and k_D in H_2O and D_2O for the Hill reaction of chloroplast fragments as defined by equation 2. The pD values were estimated by the relation pD = pH (glass electrode) + 0.4. The reaction was carried out in 0.1*M* phosphate buffer with 0.0003*M* ferricyanide at 10°C.

decreased by deuterium oxide and that the pattern of pH dependence described above becomes even more complicated. The pattern of k_D and k_L behavior in H_2O and D_2O (Fig. 5) resembles that reported by Horwitz (11), in so far as comparison is possible. The inhibitory effect on k_D is progressive and only slightly if at all reversible. Within a small experimental error the activation energy of the Hill reaction at

constant percentage inhibition is the same in deuterium oxide as in water, as shown in Fig. 6. Such a situation is incompatible with any mechanism whereby water enters directly into the activated complex for the limiting elementary reaction of k_D, since such a reaction would manifest itself in a change of activation energy accompanied by a

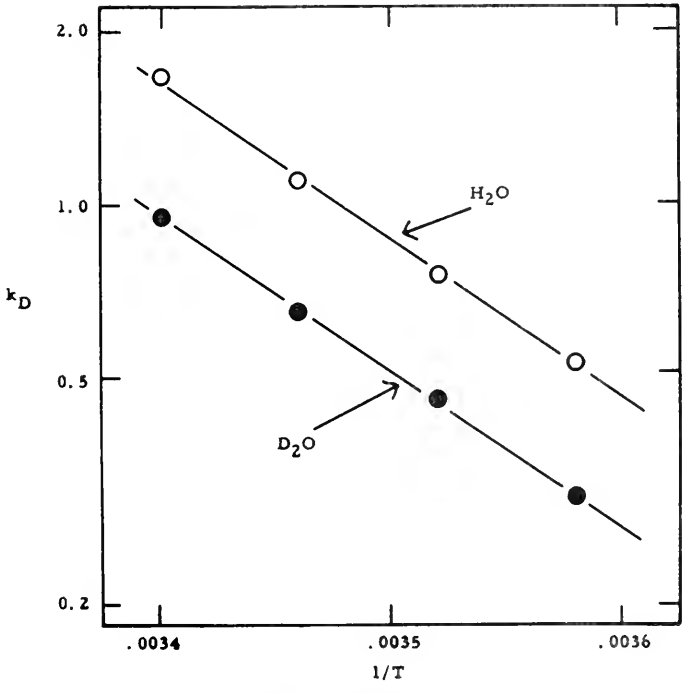

Fig. 6. Arrhenius plots of the dark reaction rate parameter k_D as measured in water and deuterium oxide over the temperature range indicated. Rates were measured potentiometrically (2) at eight light intensities. The values for the experimental energies of activation as determined by the method of least squares from the above data were: in water, 12.6 kcal.; in deuterium oxide, 12.5 kcal.

negligible change in activation entropy. We must, in general, assume that k_D consists of some combination of true rate constants for elementary steps multiplied by the concentrations of unknown "hidden" reactants. The existence of an unchanged activation energy demonstrates that water cannot be such a hidden reactant, and therefore it is reasonable to conclude that water is not involved as such in any

rate-limiting step of the dark sequence of steps under the ordinary conditions used for Hill reaction studies. Thus, deuterium oxide seems to act by irreversibly removing or denaturing some unknown hidden participant. A similar situation may exist for *Chlorella* photosynthesis, where no influence of deuterium oxide on the activation energy was found at high light intensities (10). The comparison here is interesting, since photosynthesis frequently appears to be limited at high light intensities by different slow steps from those involved in the Hill reaction. We must, therefore, conclude that there is little evidence at present which allows one to fix any elementary reaction of the overall chain as the one at which water enters.

EFFECTS OF FLASHING LIGHT ON THE HILL REACTION

The kinetic studies in this laboratory by Gilmour on the behavior of the Hill reaction of isolated chloroplasts in flashing light have been briefly reported elsewhere (13). This work demonstrated a more complex situation than was observed by Emerson and Arnold (14) for *Chlorella* photosynthesis and in so doing established the method as one of considerable power for studying the Hill reaction. The results resembled qualitatively, though not quantitatively, those of Tamiya and Chiba (15). A quantitative reconsideration of Gilmour's observations shows that they do not unequivocally support the interpretations previously made (13). Unfortunately, the experimental conditions under which our data were obtained did not permit a determination of several important facts, such as the dark time required for the ultimate maximum yield per flash. In many ways, and in fact wherever a comparison has been possible, Gilmour's data support the kinetic interpretation Kok has made of his flashing-light studies on *Chlorella* (see B. Kok's paper in this volume). The comparison is so close as to suggest that only quantitative differences due to different values of the rate constants of the two systems are involved. It must be noted, however, that both the steady-state velocity and the limiting step at high light intensity in Kok's experiments were sensitive to cyanide in low concentration, whereas we have not yet found any steps in the Hill reactions which are highly sensitive to cyanide (16).

INHIBITORS

Inhibition studies of photosynthesis have not yet reached the usefulness manifested in enzyme chemistry—a consequence of the

lack of quantitative data which would allow the determination of the method of inhibition, the thermodynamic binding quantities, and the particular elementary steps of the whole process which are influenced. A full account of our results in this area of investigation would be out of place here but several generalizations may be mentioned.

We have investigated a variety of urethanes and related compounds, metal chelating agents, sulfhydryl inhibitors, Hill oxidants, and a scattering of other powerful inhibitors, as well as a large number of less specific agents effective only at high concentration. All strong inhibitors, including the so-called narcotics which have been presumed to be nonspecific denaturing agents for proteins, give mass-law behavior with small orders of the inhibition reaction in inhibitor concentration. Failure to find exact mass-law behavior in some cases appears to be due to lack of control in the experiments and to complications introduced by the presence of Hill oxidants. For example, at high oxidant concentrations phenylurethane inhibits only k_D but experiments in the middle range of oxidant concentration show a pronounced k_L inhibition. Inhibitors differ in their effect even in the middle region depending on the oxidant employed.

Inhibitor studies are complicated by inactivation of chloroplasts during long experiments as well as by local heating under intense illumination. Most of our inhibitor results must thus be considered to be of a preliminary nature. However, some observations of a qualitative nature are valid. In general, strong inhibitors reduce both k_D and k_L though with different orders and at different concentrations. In terms of a simple geometric "photosynthetic unit," an inhibitor influencing any slow step along the chain would appear to have an identical inhibiting effect on any detectable preceding slow steps, since mass-action kinetics would not apply in a localized array of molecules. Such does not appear to be the case, and we must seek a more complicated mechanism—a conclusion which can be seen to be also a consequence of the rate law. No purely k_L inhibitors have been found except Hill oxidants, perhaps suggesting that k_L and k_D slow steps are closely related. Detailed investigation of the inhibition reactions should thus provide a means for relating these slow steps and for determining the characteristics of the molecules involved. In the few cases thus far examined, k_L inhibition is paralleled by quenching of fluorescence so that we may tentatively conclude that some in-

hibitors for the Hill reaction attack chlorophyll directly—a conclusion long since established for the whole photosynthetic process. This conclusion is not, however, trivial, for more and more differences between the two processes are becoming apparent. The latest of these manifests itself as a difference of activation energy for the k_D process. We have found that quantity to be considerably lower than the many values reported for the dark-limiting step of photosynthesis.

CONCLUSION

A wide variety of experiments on the kinetics of the Hill reaction has provided large numbers of data, some of which, it must be admitted, may be shown to apply only to the Hill reaction and not to the overall process of photosynthesis. However, Hill reaction data can be obtained under much more thoroughly controlled conditions so that in many cases they are less confused by artifacts than information of the type presently available on whole-cell photosynthesis. Not all the essential control variables are known or understood in the Hill reaction of isolated chloroplast fragments, but there appears no reason to doubt that the remaining difficulties, which are largely associated with variables in plant-growth conditions and chloroplast preparative techniques, will be worked out in the near future. In almost every respect, results with the chloroplast Hill reaction have demonstrated simple basic forms which are already well known in small-molecule reactions or in enzyme reactions. As a result it seems probable that most problems in Hill reaction kinetics will be susceptible to explanation on the basis of mechanisms already understood. Certain essential experiments have not yet been successfully completed in our studies, nor do we have sufficient data of the necessary precision on the flashing-light effects, pH, deuterium oxide effects, etc., fully to establish the mechanisms involved. The data and observations summarized in this paper can, however, be partially integrated. These interpretations along with complete details of the experiments will be published shortly.

Discussion

Spikes: The chloroplasts in the system contained about a half milligram of chlorophyll which would be about half a millimole in 3 ml. The oxidant was effective at concentrations as low as 10^{-6} M.

Lumry: May I say just one thing about the thioctic acid study of the group at

Berkeley? The thioctic acid effect seems to be superimposed on the quinone effect. The effects are additive, on the basis of their own data.

Spikes: I think I said that thioctic acid, glutathione, cystine, and a number of other sulfur-containing compounds will not function as direct Hill reaction oxidants in the system, even though they are capable of being rapidly oxidized by manganese dioxide.

Bassham: I want to ask, in view of the quantum requirement of the Hill reaction which illustrates transfer of electrons with less than 2 quanta, if you think that the process is sufficiently similar to photosynthesis to take this as an indication that in photosynthesis we also can transfer electrons with less than 2 quanta.

Lumry: The energy requirements are the same, so draw your own conclusions.

Rabinowitch: Do you think that the result obtained with extremely low concentration of oxidants is of importance in connection with Franck's hypothesis that the Hill oxidants associate themselves with chlorophyll? You have so many times fewer molecules of the reductant than of chlorophyll that there would be no chance for reactions based on association. You must have kinetic encounters in order to get a reasonable yield.

Clendenning: Was your light source white or colored?

Spikes: It makes no difference whether you do it using an ordinary incandescent lamp, so-called white light, or whether you use only a red band with the peak at 675.

Clendenning: Your effect of concentration will be exactly the same?

Spikes: It is possible. We work in a nitrogen atmosphere which cuts down the side effects. Also the blue component of an ordinary incandescent bulb is quite low. There is very little direct photochemical effect even with ordinary white light.

Brown: We have seen here several effects where the chloroplast reaction seems to behave differently from the photosynthetic in a way which might be considered fundamental. The fact that a much higher saturation rate can sometimes be obtained has certain implications, even embarrassing implications. The fact that the k_L for the chloroplast system is sensitive to heavy water, whereas what data we have on the photosynthetic system indicate that an equivalent k_L is not sensitive to heavy water, also makes one suspect that there may be a basic difference in mechanism.

Another point which has not been discussed, but which is also fundamental, is the fact that a single flash of light, a very brief flash, that will produce oxygen in the Hill reaction will not produce photosynthesis.

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Kinetics of the Photosynthetic Incorporation of Radiocarbon

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The kinetics of incorporation of radioactive carbon into various compounds in photosynthesizing *Scenedesmus* are available (1). $C^{14}O_2$ having been incorporated under conditions as near steady state as possible, one may seek to ascertain whether a qualitative correspondence may be shown with the known pathway of carbon dioxide

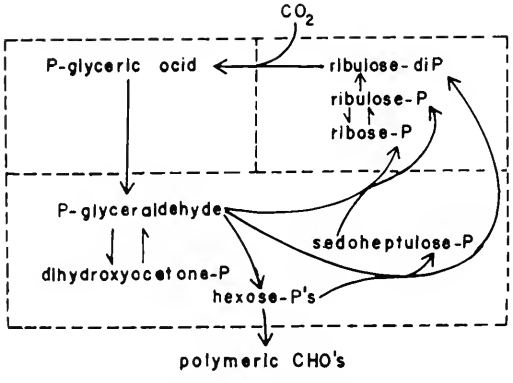


Fig. 1. Scheme for the photosynthetic assimilation of carbon dioxide in *Scenedesmus*, as proposed by Calvin and co-workers. The reversibility of all of the reactions within the cycle is presumed but not indicated. Erythrose phosphate is at present still unidentified. Recent findings also suggest the possibility of the formation of xylulose as a precursor of ribose.

assimilation (Fig. 1). The correspondence is sought in two types of curves: (*a*) the rate of incorporation of isotope into the various compounds (including its distribution within the individual moieties) and (*b*) the changes in concentrations of the compounds during transition from one steady state to another. Required parameters include a knowledge of the respective rate constants and the steady-state concentrations. Although the latter has been determined (for a par-

ticular organism under specific conditions) (2), the rate constants are generally unknown or only surmised. Consequently a quantitative comparison of theoretical and experimental kinetics is not possible at present. By the assumption of reasonable rate constants, one may derive curves which may be compared qualitatively. From Fig. 1, it is apparent that such curves would involve the solution of a

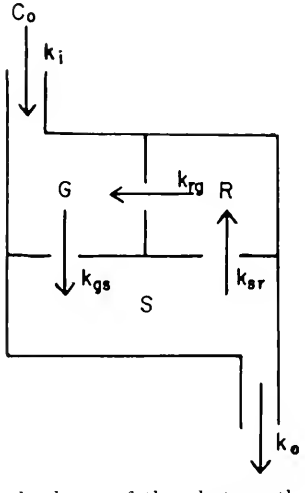
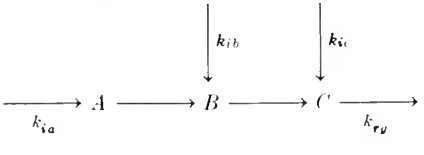


Fig. 2. Compartmentalized scheme of the photosynthetic assimilation of radioactive carbon dioxide.

set of at least eight differential equations—a computation amenable to the patience only of a differential analyzer or analog computer. The mathematics involved in radioisotope incorporation may be simplified considerably by the grouping together of various compounds, as shown in Fig. 2. In this manner, one is concerned with a three-component system and derives curves which represent the combined rates of the individual members of the compartment. In principle, one may then evaluate the individual rates by making use of the calculated combined rate constant. For example, consider compartment R, consisting of the pentose sugars in the system



where $k_{ib} = k_{ic}$ and $k_{ia} + k_{ib} + k_{ic} = k_{sr}$. One may obtain k_{sr} and k_{rg} from the calculations to be presented below. From (future) knowledge of the enzyme kinetics of the above reactions, one will know k_{AB}, k_{BA} and k_{BC}. Consequently, by solution of a pair of simultaneous equations describing dA/dt and dB/dt, one should be able to solve for k_{ia} and $k_{ib} = k_{ic}$. In a similar, though more complicated manner, the solution of compartment S may be obtained.

The formal set of differential equations describing the rate of incorporation of radioactivity into each of the compartments of Fig. 2 is:

$$\frac{dG}{dt} = C_0 k_i + k_{rg} R - k_{gs} G$$

$$\frac{dS}{dt} = k_{gs} G - (k_{sr} + k_0) S$$

$$\frac{dR}{dt} = k_{sr} S - k_{rg} R$$

where G, R, and S are specific activities of PGA, the pentose sugars, and the residual sugars, respectively. The subscripts of the various k's denote direction of movement of the isotope. If $\bar{G}$, $\bar{R}$, and $\bar{S}$ denote the corresponding steady states of G, R, S, then the set of linear, nonhomogeneous equations may be converted to a set of homogeneous equations by substitution of the corresponding differences, $\alpha = G - \bar{G}$, $\beta = S - \bar{S}$, $\gamma = R - \bar{R}$, following the methodology of Denbigh (3). The solution of these difference equations is readily shown to be

$$\alpha = \mu_{1g} e^{-m_1 t} + \mu_{2g} e^{-m_2 t} + \mu_{3g} e^{-m_3 t}$$

$$\beta = \mu_{1s} e^{-m_1 t} + \mu_{2s} e^{-m_2 t} + \mu_{3s} e^{-m_3 t}$$

$$\gamma = \mu_{1r} e^{-m_1 t} + \mu_{2r} e^{-m_2 t} + \mu_{3r} e^{-m_3 t}$$

where the m's are the roots of the cubic equation:

$$m^3 - m^2[k_{rg} + k_{gs} + k_{sr} + k_0] + m[k_{rg} k_{gs} + (k_{rg} + k_{gs})(k_{sr} + k_0)] - k_0 k_{rg} k_{gs} = 0$$

and the constants have the relation:

$$\mu_{ir} = \frac{k_{gs} - m_i}{k_{rg}} \mu_{ig}$$

$$\mu_{is} = \frac{k_{gs}}{k_{sr} + k_0 - m_i} \mu_{ig}$$

The constants may be evaluated by solution of the simultaneous equations involving α_0, β_0, and γ_0, where $\alpha_0 = \bar{G}_0 - \bar{G}$, etc. It may be shown

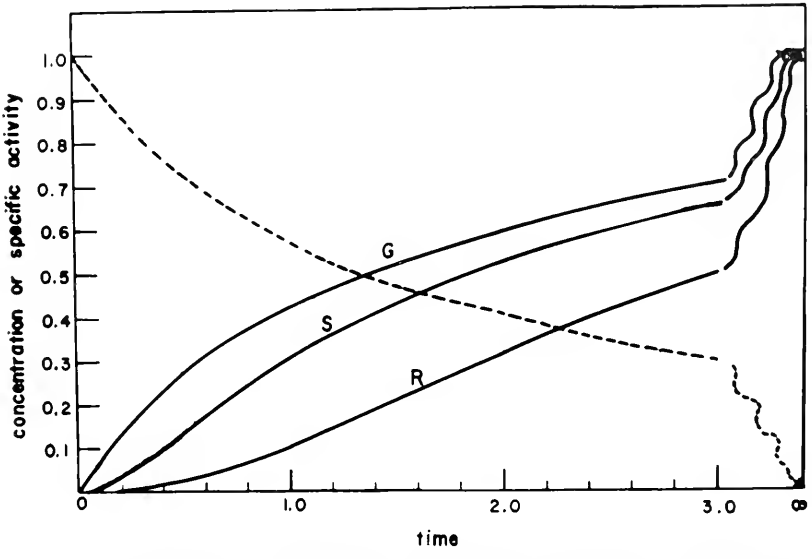


Fig. 3. The accretion of radioactivity within each of the three compartments, assuming unit values for all parameters. The dotted curve denotes the loss of radioactivity from compartment G on sudden removal of radioactivity from incoming carbon dioxide.

that the roots of the cubic equation, m_i, may be either real and distinct, or one real and two complex. If one chooses parameters of unity, i.e., $C_0 = 1$, and $k_1 = k_0 = k_{rg} = k_{sr} = 1$ (so that $k_{gs} = k_1 + k_{rg} = 2$), then the m's contain complex roots. Under these conditions the solutions of the equations for G, S, and R are:

$$G = 1 - 0.740e^{-0.301t} + 0.305e^{-2.344t} \sin(1.0288t + 4.159)$$

$$S = 1 - 0.873e^{-0.301t} + 0.563e^{-2.344t} \sin(1.0288t + 3.368)$$

$$R = 1 - 1.255e^{-0.301t} + 0.331e^{-2.344t} \sin(1.0288t + 0.880)$$

The trigonometric solutions provide a convenient method of calculation of the terms involving the paired complex numbers. The steady state is therefore approached as a difference between unity and the sum of a diminishing logarithmic and a damped periodic function. Theoretically, parameters might be chosen where the steady state is approached in a damped, oscillatory manner. However, with the parameters we have used, term three never exceeds term two and the curve is strictly monotonic, as Fig. 3 shows.

If withdrawal of isotope is established suddenly (that is, $C_0 = 0$), then the loss of radioactivity from each of the compartments is a curve equivalent to the inversion of the accretion curves.

The isotope accretion curves are, in fact, qualitatively in agreement with the experimental data (1), despite the arbitrary choice of parameters. Thus, there is the initial near-linear rate for PGA (= compartment G) and the initial sigmoid rates for the pentoses, as well as for the residual sugars. A plot of the curves as per cent of total activity vs. time also results in the negative slope for G corresponding to that found experimentally for PGA.

Under isotopic steady-state conditions, the total radioactivity per compound is proportional to the pool size of the compound. A change in one of the rate constants or the CO_2 concentration will result in transient changes, eventually leading to the establishment of new steady states. The radioactivity of the compounds may then be utilized to describe these changes, as has been shown experimentally by Wilson (4) in Calvin's laboratory. A discussion of these transients, as well as a fuller exposition of the methods employed here, is presented elsewhere.

Acknowledgment. We wish to acknowledge the generous financial support of the National Science Foundation.

Discussion

Witt: What is the time scale?

Aronoff: Arbitrary time units.

Lumry: We have worried quite a bit about the kind of system you have just discussed and have found it to be of limited usefulness because of the frequent possibility that not all elementary steps are first-order in substrate concentration. For example, an enzymatic step operating under conditions of enzyme saturation introduces a zero-order step so that an entirely different type of mathematical framework becomes necessary. Secondly, your drive for the whole mechanism is

provided from an external source in a reaction which again is not first-order in substrate concentration.

Aronoff: Indeed, it is just as valid to say that it does not hold if there are second-order reactions. Anything but the first-order would make the scheme invalid as postulated.

Myers: No, it would just reduce the number of terms.

Lumry: No, Aronoff's system is strictly limited to catenary chains of elementary reactions each of which is first-order in a product of the preceding step. If some enzyme is working to full capacity, its concentration and the rate of its unimolecular reaction yielding product will limit the overall reaction, thus acting as a bottleneck not included in Aronoff's scheme.

Bassham: I would like to make three comments, if I may.

1. We have the same kind of overshoots on turning off the light that you get with decrease in the CO_2.

2. I would have thought that if you were going to be limited to three compartments, it might have been better to have put the pentose-monophosphate in the compartment for ribulose diphosphate. The reason for this is that in the carbon-reduction cycle there are two points at which external factors are required, at least according to the present hypothesis. One is the reduction of PGA and the other is the conversion of ribulose-monophosphate to ribulose diphosphate. Another reason is that the pentose monophosphates are made by several different pathways while the ribulose diphosphates are made presumably by only one pathway from ribulose monophosphate.

3. Still another comment I want to make is that Dr. Bradley, who worked in our laboratory, has made some similar kinds of calculations and he has also had some difficulty in getting the condition for these overshoots you mentioned.

Aronoff: My interest in this problem was stimulated by the model which Bradley and Calvin used. This was, however, a linear model, not cyclic. To that extent it cannot apply to the system that we are considering. The reason that the system was divided in the way it was, apart from sheer convenience, was that the presentation of yours was in terms of the di-phosphate sugar. If you presented your data in terms of the amount of activity in ribulose diphosphate per time unit, then I would be very happy. However, there are no specific data for that particular fraction. The scheme is nevertheless valid because all the material falls into those sugars as depicted.

Bassham: Yes, but the various pentose phosphates are made by several different pathways.

Aronoff: There is a total of three different pathways going into that compartment, and all three are included in the one constant.

Bassham: Wouldn't it be better to stick to the condition where there is just one reaction, especially since in that reaction an external factor is introduced, namely ATP?

Aronoff: For analyses of this type one should have a differential analyzer. Unfortunately, I have to do this with my slide rule, or, occasionally, a borrowed calculating machine. With this equipment a three-compartment system is complicated enough.

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Transient Phenomena in Leaves as Recorded by a Gas Thermal Conductivity Meter

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The work to be reported here deals with the measurement of gas exchange rates and the determination of gas exchange quotients during transient phenomena in photosynthesis. The method of gas analysis introduced by Aufdemgarten and later used by Van der Veen for the study of photosynthesis seemed well adapted to this investigation. This method is based upon the change of heat conductivity of a gas mixture which accompanies a change of gas composition. The advantage of this method is that the sensitivity to a small change in concentration of a component is independent of the concentration of that component already present. Thus photosynthetic gas exchange can be determined in an atmosphere containing 4% CO_2 and 20% O_2 without loss of sensitivity due to these high background concentrations. In order to measure the O_2 gas exchange, gas mixtures containing He rather than N_2 were used. In an atmosphere of air, O_2 changes are not manifest, since the heat conductivity of O_2 is about the same as that of air.

The gas mixture (generally 4% or 0.04% CO_2 and 20% O_2 in He) passed over a leaf at a constant rate of flow and then through the analyzing apparatus. Since a constant flow was maintained over the leaf, the apparatus measured rates of gas exchange. The curve for the rate of O_2 exchange versus time was obtained by placing a CO_2 trap in this gas line between the leaf and the analyzing chamber. When this trap was bypassed, the combined effect of O_2 and CO_2 exchange was measured (called $O_2 + CO_2$ curve). Thus, in order to determine the curve for the rate of CO_2 exchange in the same experimental atmosphere, two experiments had to be made under identical conditions: one with and one without the CO_2 trap. The difference between the O_2 and $O_2 + CO_2$ curves gives the CO_2 curve. A typical set of curves is shown in Fig. 1.

The lag of about 30 seconds after the onset of illumination before the photosynthetic gas exchange is recorded is due to the time re-

quired for the gas to flow from the leaf to the analyzing chamber. The inertia and time delay of this system, although less than manometric measurements, preclude measurements as rapid as those of Brackett and Gaffron.

In order to determine the reading which corresponded to the compensation level on these curves (i.e., the point at which the gas exchange of respiration is compensated by that of photosynthesis), the gas flow was shunted around the leaf. With such a bypass there is no change in gas composition.

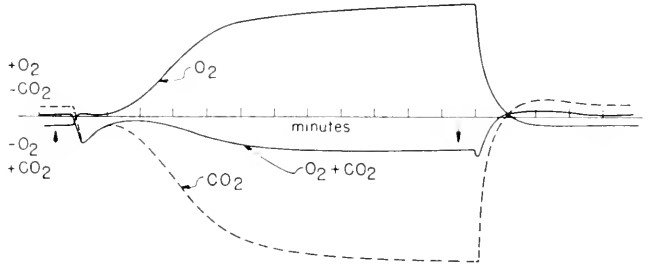

Fig. 1. Rate of gas exchange vs. time for a hydrangea leaf at room temperature in an atmosphere of 4% CO_2 and 20% O_2 in He.

Since the apparatus is somewhat more sensitive to CO_2 changes than to O_2 the displacement of the curve due to the CO_2 assimilation is greater than that due to the O_2 evolution during the steady state when the assimilatory quotient is unity.

The steady-state rate of O_2 evolution and CO_2 uptake is 18 μl/min. This changes the composition of the gas from 4% CO_2 and 20% O_2 to 3.964% CO_2 and 20.036% O_2.

In most of the work the under side of hydrangea leaves was irradiated. It was determined that the stomata are all on the under side of the leaf and are always open.

Figure 1 shows a typical set of curves obtained with 4% CO_2 and 20% O_2 in He. The initial shoulder in the O_2 curve at or slightly above the compensation level is a general feature of these curves, provided the previous dark period has been at least 5 or 10 minutes. The shoulder is independent of light intensity provided the steady-state rate of photosynthesis is somewhat higher than compensation. At low CO_2 pressures, the shoulder remains at compensation, although it becomes less prominent because the induction period is of shorter duration.

The curve for the rate of CO_2 exchange shows quite a marked dependence upon the CO_2 pressure. With 4% CO_2, as may be seen in Fig. 1, there is a pronounced uptake of CO_2 beyond the compensation level during the first minute of illumination. The assimilatory quotient (expressed as rate of O_2 evolution/rate of CO_2 uptake) during this first moment has a minimum value between $^1/_2$ and $^1/_3$. During the monotonous rise to the steady-state rate of photosynthesis, the quotient is unity.

Under conditions of low CO_2 pressure (0.04% atm.), the initial anomalous quotient is not seen. Here the CO_2 curve parallels the O_2 curve fairly well throughout the entire induction period. An accurate CO_2 curve at low CO_2 pressures is difficult to obtain, however. The method of analysis does have some inertia so that brief induction phenomena will be smeared out. The CO_2 curve obtained under conditions of low CO_2 pressure (0.04% atm.) shows a smooth, monotonous rise from the rate obtaining at the level of respiration to that at steady-state photosynthesis. It is believed, however, that there is actually a brief shoulder at the level of compensation which is not seen. The sensitivity of the apparatus required that the atmosphere be depleted by about 0.02% CO_2 during steady-state photosynthesis. Thus, if the initial concentration of CO_2 in the gas stream was 0.04%, which already markedly limits photosynthesis, there would be an increasing degree of CO_2 limitation as the gas passed over the leaf owing to the photosynthetic removal of CO_2 from the atmosphere. The curve for the gas exchange during the induction period would be further complicated since the degree of CO_2 limitation experienced by any one particular section of leaf would be increasing as the rate of photosynthesis increased toward its steady-state value. The curves obtained under these changing conditions would not be expected to show very brief induction phenomena which might be obtained at a low but constant CO_2 pressure. When the pressure of CO_2 is above 0.1% atm., so that the leaf is not subject to such a wide degree of CO_2 limitation, the shoulder is clearly observed. Gaffron's very rapid CO_2 measurements with a recording pH meter show that, with algae under conditions of low CO_2 pressure, the CO_2 exchange goes to the compensation level at the instant the light is turned on and pauses there for a few seconds before rising to steady-state photosynthesis. The measurements of McAlister and Myers with an infrared CO_2 analyzer on both algae and young wheat plants

at low CO_2 pressures show a shoulder at the compensation level. Thus we may safely conclude that at low CO_2 pressure after a dark period of a few minutes the CO_2 exchange pauses momentarily at compensation.

Fig. 2. Rate of O_2 exchange vs. time for a hydrangea leaf at 0°C. Curve *A*: 0.04% CO_2 and 20% O_2 in He. Curve *B:* 4% CO_2. Photosynthesis is running at its steady-state rate when the light is turned off.

In trying to separate the initial transient reaction from steady-state photosynthesis we found an interesting inhibition due to high CO_2 pressures. It is seen clearly when the temperature is lowered to about 0°C. In lowering the temperature from about 25°C. to 0°C.

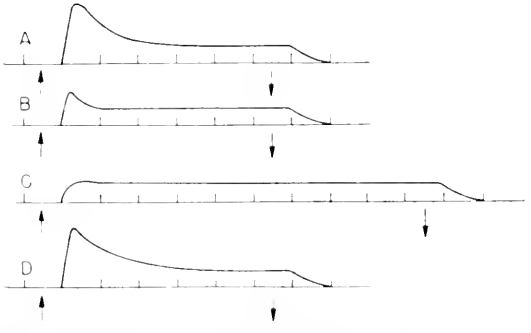

Fig. 3. Rate of O_2 exchange vs. time for a hydrangea leaf at 0°C. in an atmosphere of 4% CO_2 and 20% O_2 in He. Curve *A:* dark 10 minutes. Curve *B:* dark 1 minute. Curve *C:* dark 40 minutes. Curve *D:* dark 10 minutes.

the steady-state rate of photosynthesis in 0.04% CO_2 is reduced to $^1/_2$ to $^1/_3$ while the rate in 4% CO_2 may be reduced to $^1/_{50}$ of its rate at room temperature. In Fig. 2, the temperature was reduced while steady-state photosynthesis was proceeding in order to eliminate the possibility of exceedingly long induction effects. It is seen that the steady-state rate of photosynthesis in 4% CO_2 is about one-tenth that in 0.04% at 0°C.

Even though the steady-state rate in 4% CO_2 at 0°C. is very low, the initial induction phenomena may remain sizable. In Fig. 3 obtained at 0°C., curve *A* has had a previous dark period of 10 minutes. When the dark period is shorter, such as 1 minute for curve *B*, or longer, such as 40 minutes for curve *C*, the initial O_2 burst is smaller. Curve *D*, however, follows the 10-minute illumination period of curve *C* by a 10-minute dark period. Here the large effect has been regained. This dark period produces the maximum O_2 burst.

Under these conditions of high CO_2 pressure and low temperature, there is a CO_2 uptake associated with the O_2 burst. As shown in Fig. 4, the assimilatory quotient during this transient phenomenon is about

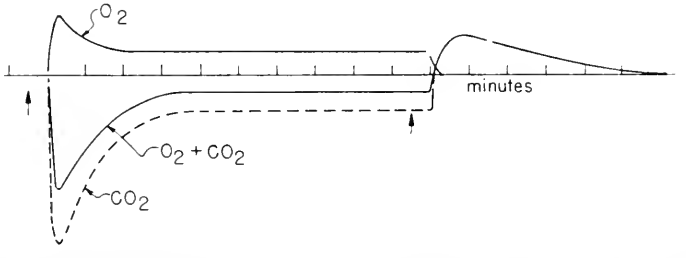

Fig. 4. Rate of gas exchange vs. time for a hydrangea leaf at 0°C. in an atmosphere of 4% CO_2 and 20% O_2 in He.

1/3. When the irradiation ceases, there is a release of CO_2 but no concomitant O_2 effect. This CO_2 release following illumination is also present at high temperatures if the CO_2 concentration is high, as can be seen by a closer inspection of Fig. 1.

If we now try to interpret the data of Figs. 1, 2, and 3 in more specific metabolic terms relating them to the known chemistry of photosynthesis, those acquainted with the literature of transient phenomena will realize that the discussion must of necessity be rather involved.

Franck and others have previously postulated that half-oxidized intermediates of respiration may be photochemically reduced when the normal photosynthetic intermediates are missing. Thus at the beginning of a period of illumination, following a dark period of sufficient duration to reduce the concentration of the CO_2 acceptor molecule, ribulose diphosphate, to its low steady-state dark value, PGA from respiration may be photochemically reduced. This will, so

to speak, "pump prime" the photosynthetic apparatus. As the process of photosynthesis gets going, it will regenerate its own PGA and respiratorily produced PGA can return to its respiratory pathways instead of being photochemically reduced. Thus light only momentarily prevents the further respiratory oxidation of PGA. The O_2 consumption in the respiratory formation of PGA from sugar is compensated by the O_2 evolution in the photochemical reduction of PGA back to triose. The photochemical reduction of any pool of PGA present at the beginning of irradiation will cause the O_2 production to exceed compensation temporarily. The respiratory CO_2 evolution should cease and no CO_2 uptake should occur until the process of photosynthesis produces the CO_2 acceptor molecule. This explains the observed shoulder in the O_2 and CO_2 curves at the compensation level. In the case of high CO_2 pressures where the CO_2 exchange shows a marked uptake initially, there must be an additional process operative.

The data obtained at low temperature and high CO_2 pressures (Figs. 2 to 4) give some clues as to the processes involved. The initial O_2 effects may be explained in terms of respiratory intermediates. Even though the rate of respiration is very low at 0°C., the concentrations of the respiratory intermediates are not necessarily small. The concentration of reducible intermediates present at the beginning of an illumination period will be indicated by the size of the oxygen burst. Thus, after a dark period of 40 minutes, which we may assume is sufficient to establish a steady-state dark condition at 0°C., the pool size of reducible intermediates is quite small, as is shown by the small initial oxygen burst in curve *C* of Fig. 3. However, respiration of sugar will continue to form half-oxidized intermediates at a low rate. During illumination, these intermediates will be reduced to phosphorylated triose. The size of the pool of newly formed triose which is formed in the light will depend upon the rate of utilization of phosphorylated triose, e.g., for the production of the carbon dioxide acceptor of photosynthesis or for storage as food in the form of hexose. After the pool of newly formed triose has been established during a period of illumination, a dark period is necessary to develop the reducible material which is responsible for the oxygen burst at the beginning of a subsequent irradiation. These half-oxidized intermediates, e.g., phosphoglyceric acid, are formed from this pool of triose by respiration, and the pool size of these intermediates in-

creases as long as their rate of formation from triose is greater than their rate of further oxidation to carbon dioxide and water. This pool size reaches a maximum after a dark period of 10 minutes. These effects may be seen in Fig. 3. Curve *A* was recorded after a dark period of 10 minutes. The pool of newly formed triose is built up to its maximum concentration during the previous illumination. This produces the maximum oxygen burst because the pool of half-oxidized intermediates is allowed to reach its maximum concentration during this optimum dark period. Curve *B*, however, which followed curve *A* by a dark period of only 1 minute, shows a very small oxygen burst because, during such a short dark period, very little of the reducible material is accumulated. On the other hand, a very small burst is seen in curve *C* because the 40-minute dark period is sufficient for respiration to reduce the pool sizes of the triose and of the half-oxidized intermediates to their very low steady-state dark concentrations. Curve *D*, however, shows that triose must have accumulated during the 10-minute illumination period of curve *C* since, following a 10-minute dark period which allowed for the formation of half-oxidized intermediates from the triose, we again obtain a large oxygen burst.

Figure 4 shows the CO_2 exchange which accompanies the O_2 exchange at low temperature and high CO_2 pressure. The primary effect on the curves of lowering the temperature has been to reduce the steady-state value of photosynthesis to a very small value without decreasing the transient gas exchange very much. As in the case of room temperature (see Fig. 1) the assimilatory quotient during the first moment of illumination has a minimum value of $^1/_3$. On darkening there is a release of CO_2 which is probably related to the initial uptake of CO_2.

The initial anomalous quotient found under a high CO_2 pressure is of interest since it indicates that the process of CO_2 assimilation during the first minute of illumination is somehow different from that at the steady state, which has a quotient of about unity. Experiments with radioactive CO_2 are currently in progress to determine what compounds result from the carboxylation occurring during this transient period. These experiments, which are being done at low temperature in order to isolate the phenomenon from normal photosynthesis, should elucidate the process responsible for the anomalous quotient.

Transient Changes in Cellular Gas Exchange

ROBERT EMERSON and RUTH V. CHALMERS, *Botany Department, University of Illinois, Urbana, Illinois*

We have completed a detailed study of the two-vessel manometric technique, giving special attention to the conditions under which this technique was applied by Warburg and co-workers to the measurement of efficiency of photosynthesis with dense (totally absorbing) suspensions of algae. Our results and conclusions are published: "Transient Changes in Cellular Gas Exchange and the Problem of Maximum Efficiency of Photosynthesis," *Plant Physiology, 30,* 505–529 (1955).

We have found that vessel pairs of the shapes used by Warburg and co-workers show evidence of differences in diffusion lag which would lead to significant errors in the application of the two-vessel method to the measurement of rapidly changing rates of gas exchange. We have been able to match vessel pairs of different design, for equality of diffusion lag for one gas (oxygen), but we emphasize that equality of diffusion lag for one gas precludes equality with respect to a second gas of different solubility. This is an unavoidable limitation on the application of the two-vessel method to the study of changing rates. However, by matching vessel pairs for diffusion lag with respect to oxygen, it becomes possible to predict the magnitude of error to be anticipated from the inequality in lag with respect to carbon dioxide, and vessel volumes can be chosen to limit this error.

By such application of the method, we have studied transient changes in gas exchange of two species of *Chlorella*, and one of *Scenedesmus*, under conditions widely used for measurement of maximum quantum efficiency of photosynthesis. With different algae, we found great differences in the pattern of transient gas exchange. Measurements with *Chlorella pyrenoidosa* confirmed the findings of Emerson and Lewis and of Brown and Whittingham with regard to carbon dioxide "burst." There was sometimes evidence of an oxygen "burst" in the light, and in general there was evidence of a maximum in respiratory oxygen consumption during the first minutes of darkness.

If the quantum requirement for oxygen production was calculated from the maxima in light and darkness, values of 3 or 4 quanta per oxygen were commonly obtainable. The evidence indicated, however, that these maxima were probably not representatives of synthesis of carbohydrate from carbon dioxide and water, but were more likely the result of changes in the size of pools of metabolic intermediates.

Calculations based on steady-rate gas exchange led in general to maximum efficiencies which represented no less than about 8 or 10 quanta per molecule of oxygen.

Discussion

Emerson: I should like to ask Professor Brown whether the mass spectrometer has confirmed the maxima for oxygen?

Brown: I have never seen the O_2 peak in the light. Although, when Whittingham and I were doing this work, we looked at the CO_2 much more than at the oxygen, we never observed it for oxygen.

Emerson: I believe that while it may soon be superseded by better and more convincing methods, the evidence from manometric measurements is indicative that for a few moments in light there may be a peak in oxygen production.

Brown: I have two questions. First, can you specify conditions under which you can reproduce the oxygen burst? I have had the impression that you can write the prescription for the CO_2 burst, but not nearly so easily for the oxygen burst. Is this correct?

Emerson: You are right. We have less experience with the oxygen burst. I don't think, though, that it would be difficult for us to write the prescription for it as precisely as we have for the carbon dioxide burst if we gave a little more attention to it.

Brown: My other question is, what is the half-time for equilibration for a single gas under conditions of shaking, and so on, in these experiments you showed us?

Emerson: If we don't put cells in the vessel, just phosphate buffer and gas, our best measurements indicate that the half-time is around 15 seconds. With the cell suspension, however, the half-time appears to be 55 seconds or so. Part of this may be a matter of induction, although I am inclined to doubt it. I think for the the most part this 55-second half-time represents the sum total of the physical lag of the system.

Brown: Have you tried it with dead cells or with poisoned cells, so that it is strictly a physical measurement you make?

Emerson: Yes, it has been done. The lag remains nearer 55 than 15 seconds.

Gaffron: You mean this minute is not the total time but the half-time?

Emerson: Yes. It is a surprisingly long half-time.

Gaffron: I think you have reported that there is a burst of oxygen but also a quick uptake of CO_2. So we have a burst of photosynthesis. Considering the half-

time of a minute, do you think this could be first only a burst of oxygen and an uptake of CO_2 much later? It makes a great difference in the theory whether you have only oxygen first or both oxygen and CO_2 simultaneously.

Emerson: The measurements certainly indicate the CO_2 maximum to which you refer, but it appears for only a single minute, and is therefore established with much less certainty than maxima which last two or more minutes.

Arnold: The throat of the two vessels that Emerson and Lewis designed is the same, with a larger gas space in one vessel.

Emerson: All these experiments were done with that same type of vessel and equal amounts of fluid in equally shaped lower portions were shaken in vessels with different overhead gas volumes.

Kok: We have made some determinations of the equilibration of CO_2 and oxygen in a few vessels and there might be about a twofold difference between the two gases.

Whittingham: In half-time?

Kok: In half-time. I want to ask you whether you have calculated quantum yields on your pressure changes. Do you find an (apparent) quantum yield of 1 for your optimum carbon dioxide bursts, i.e., is the effect of the same magnitude as found by Warburg?

Emerson: If you calculate as he did from the maximum of respiration in the dark and maximum in the light, making some allowance for diffusion, you can calculate without difficulty, a quantum requirement of less than 1.

Induction Phenomena in Photosynthetic Algae at Low Partial Pressures of Oxygen

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An electrochemical method ("Hersch" meter) has been used to determine the time course of oxygen production in photosynthesis for partial pressures of oxygen between 10^{-4} and 10^{-1} mm. Hg. Oxygen is adsorbed at the silver cathode forming hydroxyl ions in

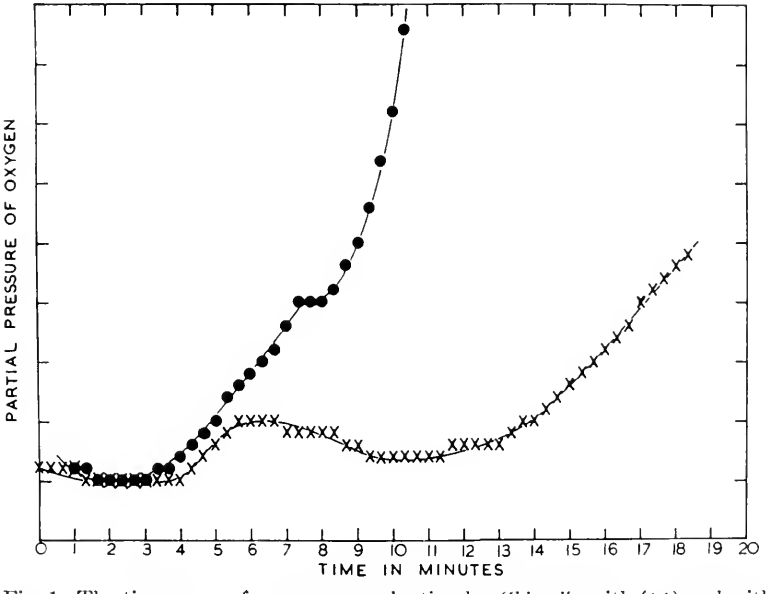

Fig. 1. The time course for oxygen production by *Chlorella* with (×) and without (●) 1.2 10^{-4} *M* *p*-chloromercuribenzoate. The graph shows the initial burst (considerably lengthened by the slow response system used here) which is depressed by the inhibitor.

solution; these oxidize the lead of the anode, causing a current to flow when the electrodes are connected by a suitable resistor. Measurements were made with *Chlorella pyrenoidosa* and *Scenedesmus*

obliquus, strain D_3, suspended in acid or alkaline media and illuminated with "saturating" intensities.

With the lowest partial pressures of oxygen used the time course separated into two phases: an initial burst followed by a subsequent rise to the steady state. This was first quantitatively investigated by Franck, Pringsheim, and Lad (1) at yet lower partial pressures. In our experiments the separation of the two phases in time was greater the lower the concentration of carbon dioxide and the longer the preceding dark period. The initial burst of oxygen, in contrast with the steady-state production, was not inhibited by 10^{-3} *M* iodoacetamide. In contrast with the Hill reaction, as measured after addition of quinone, the initial burst was inhibited by 10^{-4} *M* *p*-chloromercuribenzoate (see Fig. 1). Concentrations of fluoride and 2,4-dinitrophenol which had no effect on steady-state photosynthesis

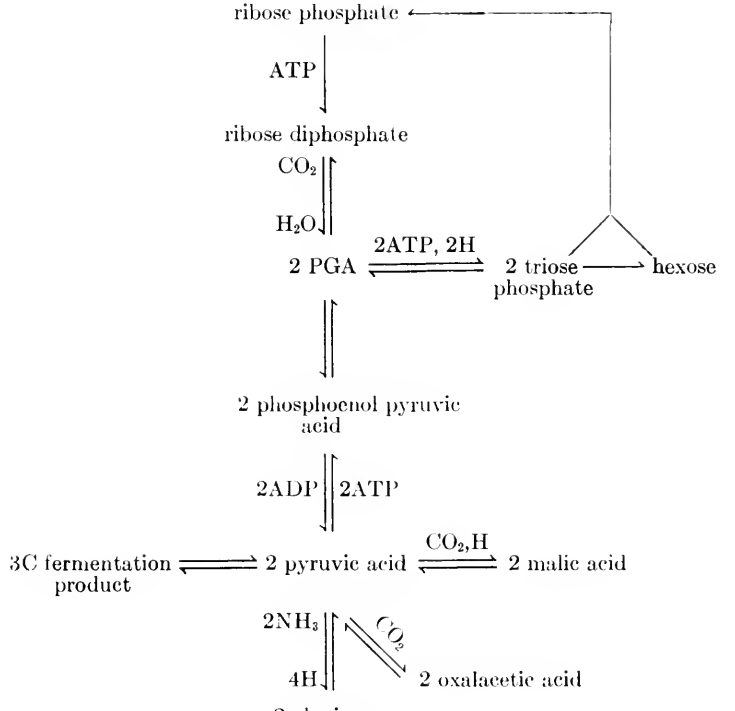

Diagram I

shortened the half-time for the attainment of the steady state, removing or at least obscuring the initial burst. The effect of fluoride was observed only if added at the beginning of the preceding dark period and not if added immediately before illumination.

The data are consistent with the views of Calvin *et al.* if we suppose that the oxygen production of the initial burst is related to reduction of PGA by some path other than reduction to triose, e.g., formation of alanine, malic, or oxalacetic acids. (See diagram I.)

Reference

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Transients in O_2 Evolution by *Chlorella* in Light and Darkness

I. Phenomena and Methods

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This paper and the one following will present studies of "early transients" in photosynthesis by *Chlorella*. These observations have been made possible by the development of an electronically recording instrument which measures both the concentration of oxygen and the rate of change of concentration.

"Early transients" seem to have a wide variety of meanings with times ranging from milliseconds to hours, depending on the time resolution of the instruments employed. Our instrument provides discretely new values every 3 seconds, in contrast to 10-second intervals in our earlier work. Furthermore, the evaluation of slope depends upon the difference of two adjacent values where formerly we required the tangent to three points. Thus our time resolution is improved threefold for concentration and nearly sevenfold for rate. Whereas rotating or flowing types of electrodes provide still more rapid response (1), we have found them nonlinear and capricious (2), as others have also reported.

Our method of oxygen determination depends as before (2) on imposing an alternating square potential pattern on a static platinum electrode and recording the current for a short period just at the end of the negative phase. Tests show that the shorter cycle still results in independent values for each point and linear dependence of magnitude on oxygen concentration.

A typical pen recording of concentration is shown above in Fig. 1, with periods of increase and decrease corresponding to light or dark. A simultaneous recording of slope or rate is obtained by a second pen writer as shown below. The background "noise" or uncertainty in

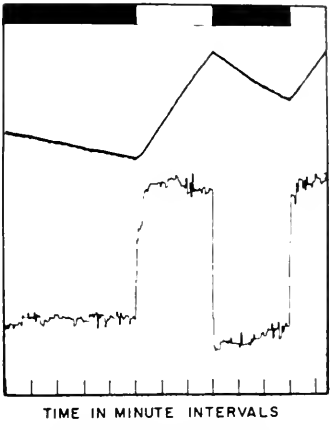

Fig. 1. Upper recording: oxygen concentration record near air saturation; normal gain. Lower recording: rate of oxygen concentration change recorded simultaneously at normal gain.

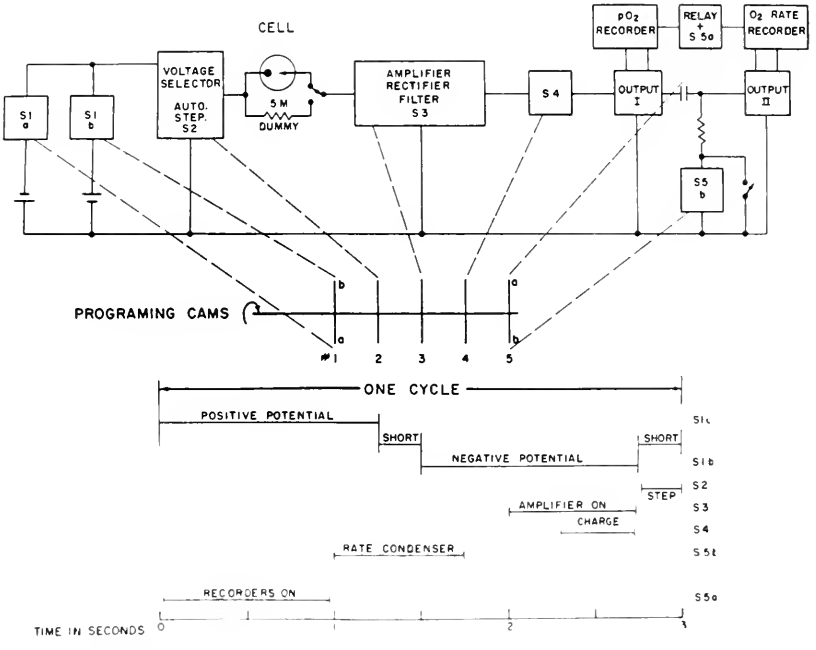

Fig. 2. Block diagram of circuit and programing cam sequence

slope has actually been improved and the "personal equation" eliminated as well.

A block diagram of the system is shown in Fig. 2. The discrete or stepwise character of the method presents novel problems in recording. A composite timing unit with five cams provides the answer.

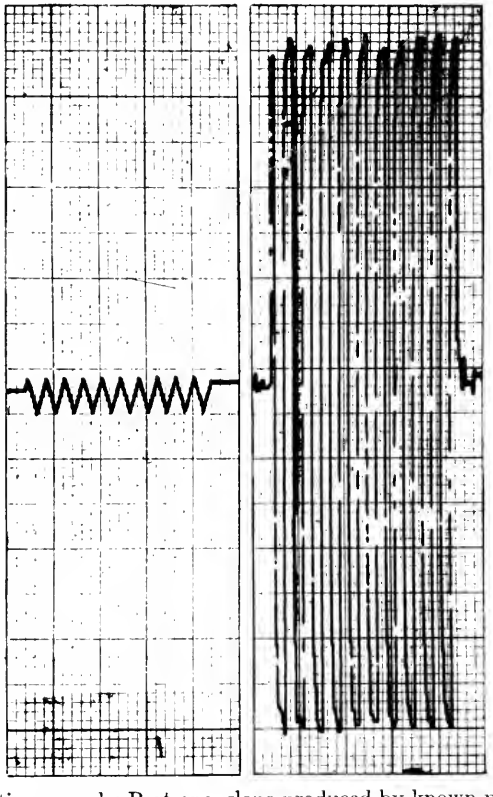

Fig. 3. Calibration records: Part one, slope produced by known potential change from stepping switch. Part two, corresponding rate record.

Thus it (*1*) generates the square potential pattern, (*2*) initiates time control of stepping switches and also of light, (*3*) cuts off the amplifier during the period of possible overload, (*4*) connects the grid condenser of the first output stage during the terminal portion of the negative phase, (*5*) grounds the grid condenser of the second output tube for a part of each cycle so that it responds only to the change, and

(*6*) turns on and off the recorders so that they respond only to the steady value attained in each cycle.

A slope recorder must, of course, be calibrated. For this purpose a stepping switch produces a known pattern of potential as shown in the first portion of Fig. 3 by the first recorder. The corresponding slope record from the second recorder is shown in the second part of Fig. 3.

METHODS

Chlorella cultures were grown aseptically as in (3) but in "lollipop" vessels provided with more homogeneous bilateral illumination. Cell concentrations in

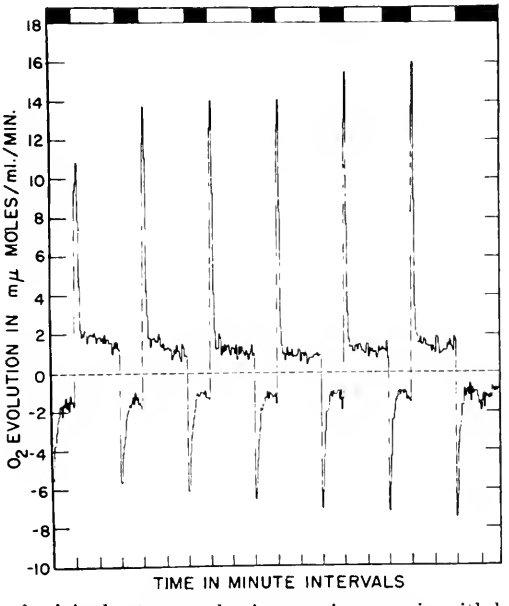

Fig. 4. Portion of original rate record using maximum gain with low CO_2 sharply limiting the sustained rate of O_2 evolution.

all cases were 2μl. cells/ml. (0.2%). Illumination of the polarographic cuvette was accomplished by a D. C. operated General Electric AH-6 mercury arc through a double Littrow monochromator set at 5780 A. The incident radiant power was in the range of 500 to 600 microwatts and hence far below the saturating level.

Comparison of our present records with those of our earlier photographic method (3) reveals little new under normal conditions. How-

ever, when the cells are observed under limiting CO_2 pressure (i.e., corresponding to less than 0.5%), one observes sharp initial bursts such as shown in Fig. 4. However, these appear not because of enhancement but because of the reduction in the subsequent sustained

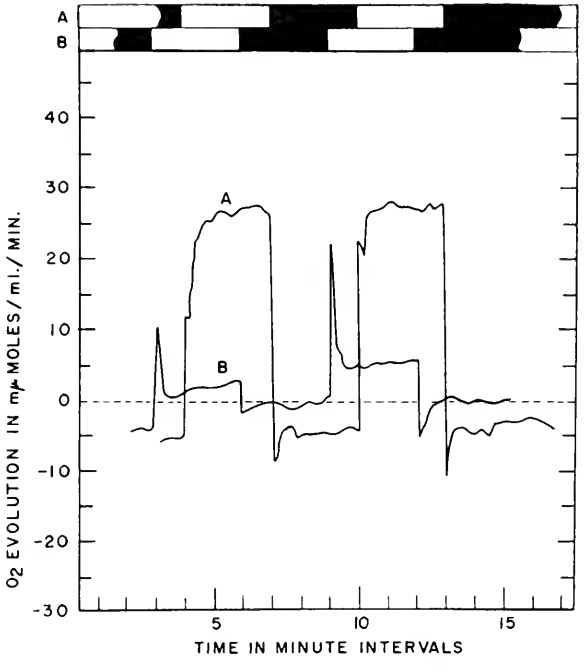

Fig. 5. Comparison of rate burst at high and low CO_2 concentration after dark adaptation for over 2 hours. Tracings of slope records: (B) equilibrated with air (0.03% CO_2); (A) (displaced) equilibrated with 5% CO_2 in air (normal gain).

rate. Thus as shown in Fig. 5,* comparing high and low CO_2, the sustained value is higher than the burst.

On closer examination, evidence of the burst is still found at the higher nonlimiting level of CO_2. This resolution of the burst from the subsequent sustained rate is partly due in this case to induction produced by several hours of dark conditioning. Of particular interest is

* Original records are shown wherever possible but tracings are used whenever intercomparison of two or more records is required.

the fact that the magnitude of the burst both with and without CO_2 limitation increases with recovery from dark conditioning, thus paralleling the disappearance of induction.

In order to see the time course of this transient disclosed at low CO_2, Fig. 6 has been plotted on an expanded time scale. The rise to maximum occurs in 6 to 9 seconds and the decay in 20 to 30 seconds.

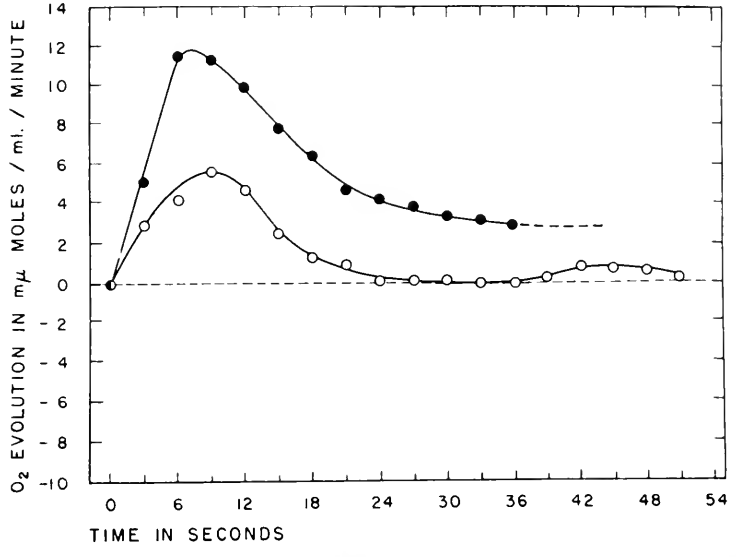

Fig. 6. Plot of initial O_2 burst at low CO_2 on expanded time scale from original recordings at maximum gain. Upper curve, after 3-minute dark period; lower curve, after extended dark period.

In the dark, an opposite burst of oxygen uptake is found. This was just barely observable in our former recordings, being shown only by the first one or two points. With better time resolution these are boldly defined. The slower fluctuations emphasized in our earlier papers (3,4) are confirmed with the background "noise" reduced to a relatively small fraction.

In a following paper we will discuss other factors influencing the initial transients and the relationship of our observations to those of others.

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3. Brackett, F. S., Olson, R. A., and Crickard, R. G., *J. Gen. Physiol.*, *36*, 529 (1953).
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II. Influence of O_2 Concentration and Respiration*

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Of the O_2 transients presented in the previous paper, attention is centered here on the oxygen burst and its relation to the subsequent CO_2-limited sustained rate. Through a study of the influence of various factors, a distinct separation of the identity of these phenomena can be shown. This may lead eventually to a clarification of the stepwise events occurring in *Chlorella* following the dark-light transition and their relation to respective reservoirs of metabolic intermediates. This information, not available from parallel studies of steady-state phenomena, should constitute a substantial contribution to the analysis of the overall process of photosynthesis.

Although concrete evidence for oxygen bursts in photosynthesis has been reported previously under anaerobic or partially anaerobic conditions (1–5 and others), determination of the factors which affect their magnitude has not been provided because of the qualitative nature of the data (1) or because of the obscuring effect of time lag introduced by the presence of air-liquid interfaces (2–4). Even in those methods which overcome these difficulties (5), the lack of derivative recording and the need of manual rate determinations attenuates the adequate quantitative comparison of burst maxima under various experimental conditions. The reduced gain required to measure such effects in aerobic suspensions offers, in this respect, even further difficulty. The advantages of quantitative polarographic rate recording are thus emphasized in the findings reported below.

METHODS

Cells were cultured and illuminated as described in the preceding paper. Cell suspensions 0.2% were equilibrated with various gas

* A preliminary report of this work was presented at the September 1955 meeting of the Society of General Physiologists. An abstract is published in *J. Cellular Comp. Physiol.*, *46*, 2, 353–354 (1955).

mixtures in water-jacketed tonometers provided with two 3-way capillary stopcocks such that air could be removed by evacuation prior to equilibration and the suspension finally introduced through a nylon capillary tube into a cuvette previously swept with the gas mixture. Experience showed this could be done without measurable contamination by air.

RESULTS

The anaerobic dark treatment of *Chlorella* suspensions provides ideal conditions for the rate recording of transient oxygen evolution.

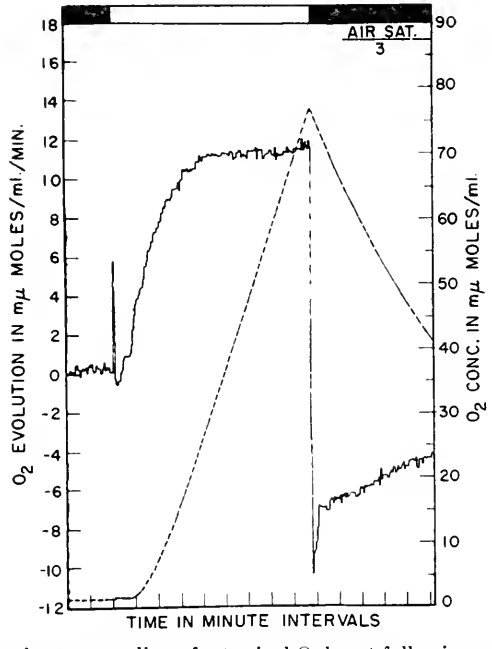

Fig. 1. Original rate recording of a typical O_2 burst following anaerobic dark adaptation, with accompanying O_2 concentration curve dotted in.

The long delay of sustained oxygen evolution (induction) by sufficient anaerobic time eliminates the complex changes resulting from a burst superimposed on a changing background rate. It also allows the use of full gain of the rate-recording amplifier without overloading by the oxygen concentrations prevailing at aerobic levels. Such a

burst rate is shown in Fig. 1* with the accompanying oxygen concentration changes. Its duration (3 seconds maximum) is shorter than bursts shown in the previous paper and, following a short interval of oxygen uptake, a secondary burst appears partially obscured, in this case, by the initiation of the sustained rate. This provides quantitative improvement over the description of anaerobic O_2 bursts previously reported by others where such details are obscured by diffusion delay and other causes. Other examples of this time course

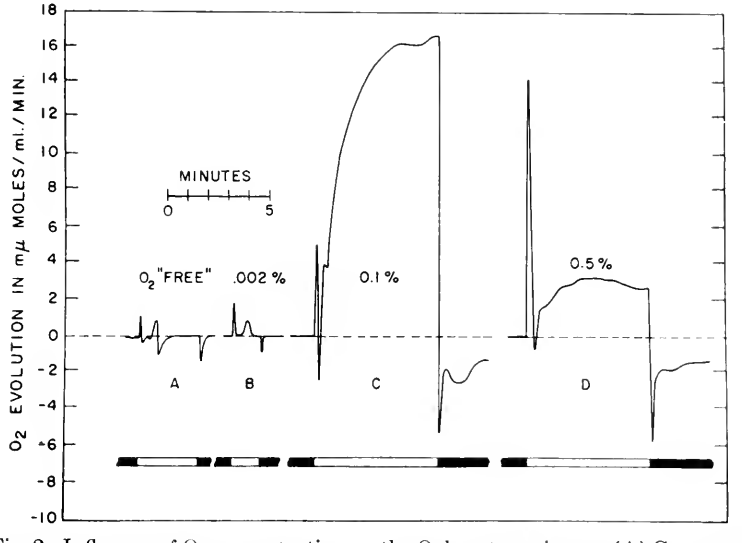

Fig. 2. Influence of O_2 concentration on the O_2 burst maximum. (A) Commercial "Seaford" grade N_2 after passing over hot reduced copper; (B) "Seaford" N_2 (0.002% O_2); (C) commercial mixture 5% CO_2 and 95% N_2 (0.1% O_2); (D) commercial water pumped N_2 (0.5% O_2).

show complete development of the secondary burst and its following period of oxygen uptake by longer anaerobic treatment and are included among the subsequent figures (Figs. 2, 4, 7, 8). Variability in the magnitude of these effects in preliminary experiments was found to depend upon oxygen concentration. In Fig. 2 the magnitude of the bursts is compared under different oxygen concentrations available with convenient gas mixtures. The maximum of the burst appears to

* Original records are shown wherever possible but tracings are used whenever intercomparison of two or more records is required.

be limited below 0.5% oxygen and becomes barely detectable at this light intensity in gases from which oxygen has been removed by hot copper. The curvilinear relationship in this range is shown by dependence on successively increasing oxygen concentrations in Fig. 9.

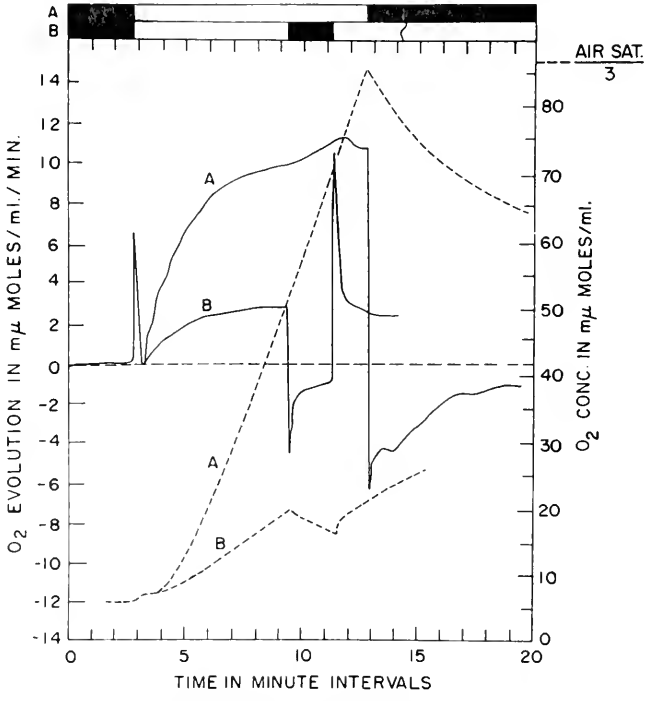

Fig. 3. Influence of reduced CO_2 concentration on anaerobically adapted suspensions. (A) with CO_2; (B) without CO_2; (A) obtained from suspensions used in B after equilibration with 5% CO_2 and 95% N_2. O_2 conc. = 0.1% at illumination in both.

Three of the recordings in Fig. 2 were made in the absence of CO_2, yet in these and other similar experiments the character of the burst is unchanged. Figure 3 shows the results with and without CO_2 in identical suspensions illuminated at similar O_2 concentrations after similar anaerobic dark treatment times. Here the sustained rate is depressed in the absence of CO_2 while both the magnitude and the character of the burst remain as unchanged as in the more aerobic CO_2-

limited bursts in the previous paper. Since the amount of residual CO_2 present from previous dark respiration is far less in this case where the bulk of respiration is anaerobic (in contrast to the aerobic CO_2-free bursts presented in the previous paper), any question of the role of residual CO_2 (see also Figs. 4 and 8) in the burst process is thereby minimized. This CO_2 independence of the burst distinguishes the process from that of CO_2 fixation.

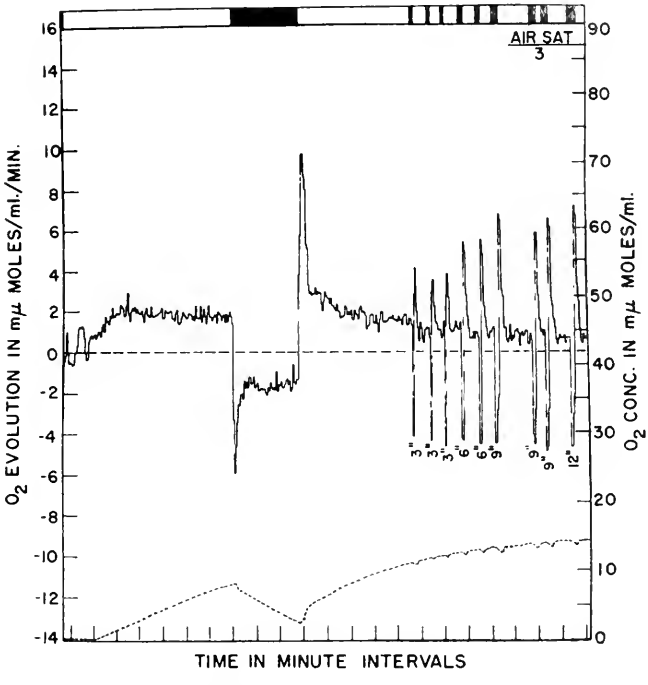

Fig. 4. Effect of interposed dark intervals during CO_2-limited sustained rate. (Portion of original rate recording with O_2 conc. curve dotted in.) Suspension anaerobic dark adapted in "Seaford" N_2 (0.002% O_2) prior to run.

Figure 4 shows the effect of interposition of successive short dark intervals of increasing length during the course of CO_2-limited sustained rate. The resulting curvilinear increase of the O_2 burst maximum with increasing dark oxidation time is plotted in Fig. 5, which shows the burst response saturating from 18 to 180 seconds.

In apparent contrast the effects of dark time on CO_2-nonlimited

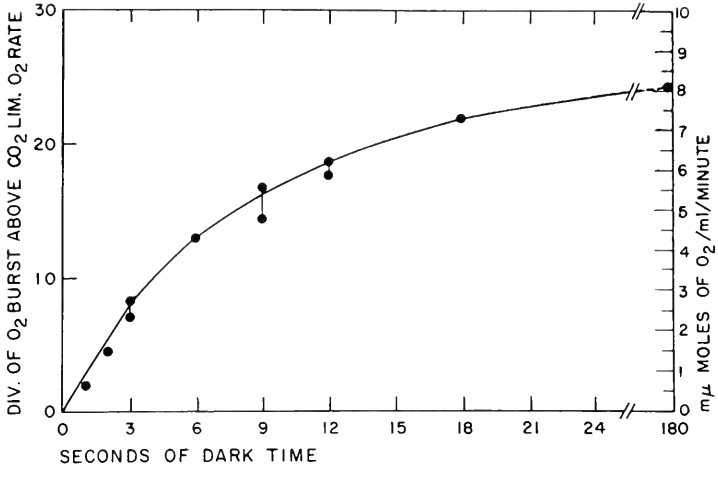

Fig. 5. Plot of data from experiment recorded in Fig. 4.

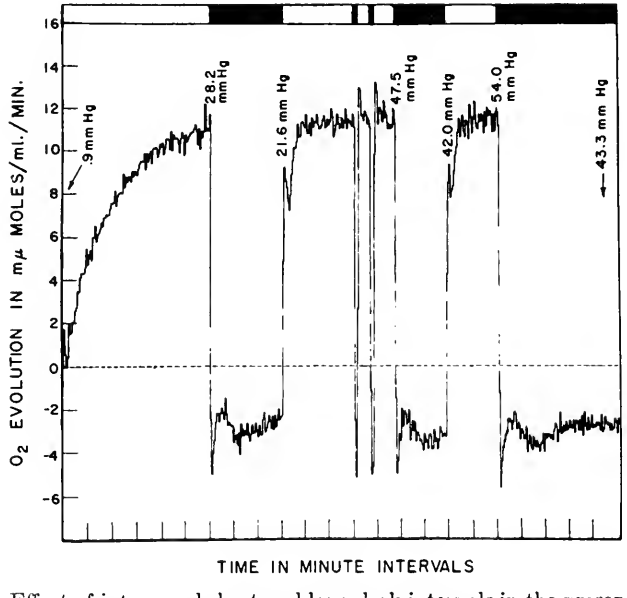

Fig. 6. Effect of interposed short and long dark intervals in the presence of 5% CO_2 (original rate record). Same conditions as in Fig. 5 but equilibrated with 5% CO_2 prior to run. Oxygen concentrations in millimeters of Hg are indicated at various points along the record.

suspensions are shown in Fig. 6. Here the superposition of the burst upon the very slight induction delay of the maximum sustained rate by dark periods of 6 to 9 seconds causes a momentary overshoot. Longer dark periods (2 minutes or more) delay the sustained rate sufficiently to partially resolve the burst at lower magnitude, as shown in the previous paper by much longer dark treatment (2 hours). This further distinguishes the burst reaction from CO_2 fixation.

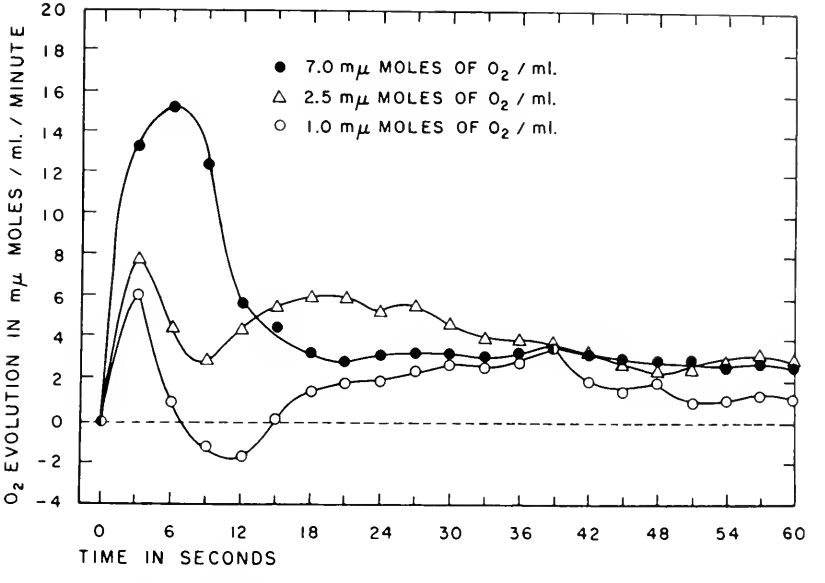

Fig. 7. Typical successive O_2 bursts occurring at increasing O_2 concentrations showing disappearance of the secondary maximum. First ○, second △, and third ● bursts.

Attention has been called above (Figs. 1 and 2, etc.) to the short duration of the burst maximum when oxygen limited. Plotting, with extended time scale, successive oxygen burst rates occurring at increasing oxygen concentrations shows, detailed in Fig. 7, the resulting progressive change leading to the disappearance of the secondary maximum. By choosing proper conditions of light intensity and time ratio of successive dark and light intervals, the transition stages of this effect may be recorded (Fig. 8) and studied in detail. They are characterized by a gradual increase of the secondary maximum leading to

an apparent fusion with the first. Attention is directed to the occurrence of this fusion only after the preceding dark oxidation rate reaches a sustained terminal value and hence above the critical O_2 concentration for oxidative respiration. The sequence of burst maxima, intervening minima, and corresponding terminal dark oxidation rates

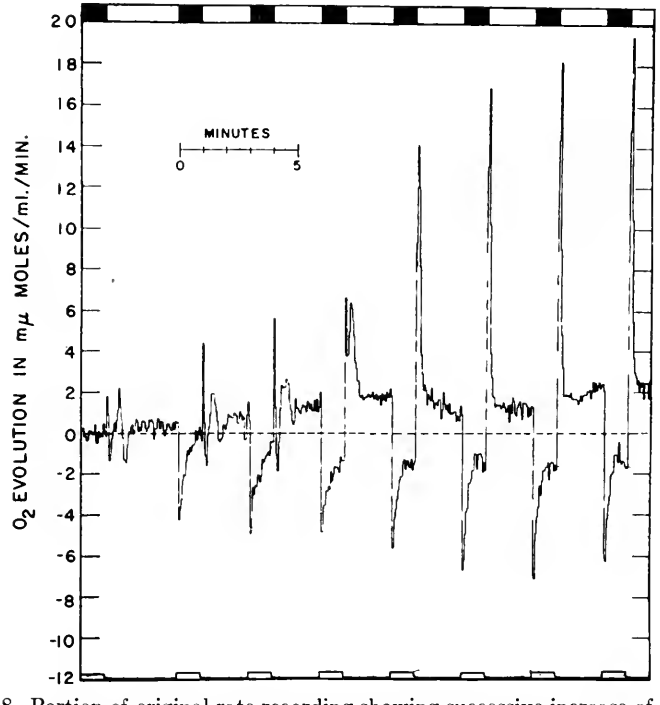

Fig. 8. Portion of original rate recording showing successive increase of secondary maximum and its eventual fusion with the primary maximum. Suspension dark adapted in Seaford N_2 (0.002% O_2) prior to run.

is plotted against O_2 concentration in Fig. 9. Whereas this indicates that the role of dark oxidative metabolism in the O_2 burst effect is not a simple one, the present state of our knowledge does not warrant its further discussion here.

DISCUSSION

The evidence, from the data presented above, for the dependence of the oxygen burst rate upon O_2 concentration (or the immediately

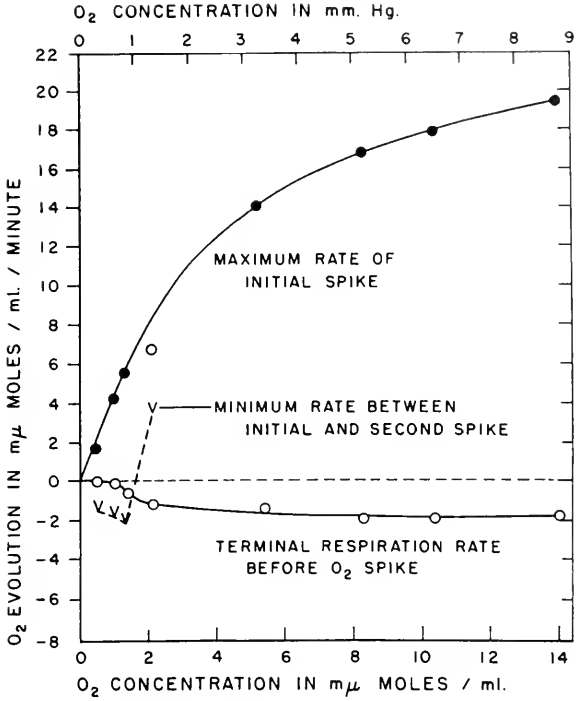

Fig. 9. Plot of data from Fig. 8.

prior dark oxidation rate) and the dissociation of the O_2 burst process from that of CO_2 fixation is harmonious with most of the available time course measurements of related processes. For example, the inverse oxygen influence on the time course of anaerobic fluorescence reported in *Chlorella* (6) is quantitatively in harmony with the burst O_2 dependence. While the range of oxygen dependence here is similar (0 to 1% O_2), further correlation is subject to the usual dependency of time course phenomena upon conditions of culture, age, etc., in *Chlorella*. Similarly, the general character of the time course of the luminescence (7) which reflects reversal of the overall process, and the time course of bound phosphate (8) approximate the composite transient O_2 rate (burst superposition on sustained rate) presented in our data. ATP and fixed $C^{14}O_2$ (9) approximate the course of our composite O_2 rate minus burst rate. In the study of CO_2 transients in *Chlorella* with the glass electrode (10,11), an interval (0 to 60 seconds)

of no CO_2 uptake is found immediately following illumination. This offers additional evidence for the dissociation of the oxygen burst process from that of CO_2 fixation.

In a recent report (5) a form of convective polarography is used to measure marked O_2 concentration changes occurring immediately on illumination of *Chlorella* suspensions under anaerobic conditions (0.001% O_2). The report offers the conclusion, viz., that the beginning of photosynthesis does not require oxygen, since the cells contain a substance which in light is capable of releasing a limited amount of oxygen, and that the sustained rate of photosynthesis is initiated by respiration stimulated by this oxygen. Attention is called to the fact that this work is subject to the quantitative limitations of convection polarography (12–14) and the condition of saturating light intensities in the presence of O_2 (0.001%). We find it difficult to reconcile the above interpretation with our finding of marked dependence of the initial O_2 burst upon oxygen concentration (or the immediately prior dark oxidation rate). Moreover, no O_2 dependence of sustained rate is indicated in (15).

In a later publication further correlation of our findings with other related phenomena will be considered.

Acknowledgment. The authors express appreciation of the technical assistance of Mrs. E. Engel, both in the experimental procedure and the culture of *Chlorella.*

Discussion

Bassham: In our closed gas circulating system, with an instrument which measures a specific property of oxygen, paramagnetism, and another instrument which measures CO_2, so that the two are independent of each other, we have observed transients in both oxygen and carbon dioxide. I cannot remember any detailed results because we have not made a systematic study of the effects, but I can say that these transients vary not only in their size but also in their direction, depending on light intensity and previous condition of the plant and many other factors. I simply mention that there are some transients in both oxygen and carbon dioxide. I cannot say more at this time about what their size and order are.

Rosenberg: I wonder if you could review for me how excessive the initial respiration was in the first instant of dark after a normal preceding period of photosynthesis.

Brackett: It very commonly doubles. In other words, the first spike is perhaps double the sustained rate of respiration.

Rosenberg: And in the inhibited cell sometimes it went to 3 or 4?

Brackett: Oh, yes, many times, but only in the inhibited cells.

Wassink: It seems to me that the picture you find in the time course, similar to the first outburst of fluorescence, is very much exaggerated at low concentra-

tions of oxygen. I wonder if you also have it at a very much higher concentration. We find that this fluorescent outburst definitely decreases very much. Is that also true of your oxygen outburst?

R. A. Olson: I think the last figure shows these fluorescent effects.

Brown: I want to make sure I did not misinterpret something by being amazed at the evidence of these data. Your spike never does exceed the maintained steady-state rate?

R. A. Olson: That is right. The spike per se only closely approaches it.

Brown: This is not reconcilable with the manometric data which were discussed previously.

Brackett: Not unless the manometric data were obtained under conditions which depress the sustained rate.

Emerson: The conditions are extremely different, since here in Dr. Olson's experiment there are hardly any sustained rates at all. The manometer is usable for longer periods; in fact, for indefinite periods of time.

Brackett: I am glad you said that. We talk about sustained rates as those that follow and are influenced by CO_2. From your standpoint, these are quite short-term phenomena.

Rosenberg: Do you know the number of micromoles of oxygen consumed in this valley between the spikes as related to the amount of chlorophyll present? This might help us in comparing the luminescence state tomorrow.

Brackett: We have made rough calculations. It is very small. Even the first burst under this more limited condition requires only a small fraction of the chlorophyll molecules.

Rosenberg: But that is the burst size?

Brackett: That is the burst size. The depression is much smaller. I thought you were asking about the negative reaction.

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Transients in the Carbon Dioxide Gas Exchange of Algae

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At the first Gatlinburg meeting three years ago, I showed some induction curves for carbon dioxide obtained with a Beckman commercial pH meter, a glass electrode, and a Brown recorder. A glass electrode registering acidity changes caused by carbon dioxide was used years ago by Blinks and Skow (1). Rosenberg in the meantime has published a paper describing within what limits our method permits the quantitative measurement of the carbon dioxide exchange in thin suspensions of algae (2). Our original purpose was to supplement the manometric method with a fast and automatically recording one, to see whether the strange phenomenon reported by Warburg and Burk, known as "the one quantum process," really existed. We found no trace of it, in best agreement not only with our own experiences throughout many years but also with the results of Brackett and Olson, Brown, Whittingham, Calvin and, of course, Emerson. Our efforts in this respect have been published (together with a list of references) and there the matter may rest (3).

Fortunately it turned out that this very sensitive, reliable, and fast method revealed a number of new aspects of the induction period and other transients for carbon dioxide which have not been discussed before.

I shall show a few selected curves, particularly those which invite direct comparison with Brackett and Olson's transients for oxygen ((4) and pages 412–418, this book) and with McAllister and Myers' old induction curves for carbon dioxide (5).

To forestall some obvious questions we contrast (Fig. 1) *acidity changes during transients* as found in phosphate buffer lacking carbon dioxide with those seen in bicarbonate buffer. At pH 6.9 in phosphate buffer with little free CO_2, the algae seem not to notice much whether the light is being turned on or off. If organic acids other than carbonic are being suddenly released into, or suddenly reabsorbed from, the

medium, the amounts are too small to register under our conditions. Presence of carbon dioxide either in water or in diluted neutral phosphate media, or in pure solutions of mixed sodium and potassium carbonates, on the other hand, leads to conspicuous changes in the recorded pH upon illuminating or on darkening.

Thus we proceed under the assumption that our apparatus responds mainly to those pH changes which are due to a metabolic carbon dioxide exchange. A release of CO_2 by respiration or fermentation lowers pH and the recorded curves in our figures slope up. Photosynthesis increases pH and the curves slope downward. A horizontal

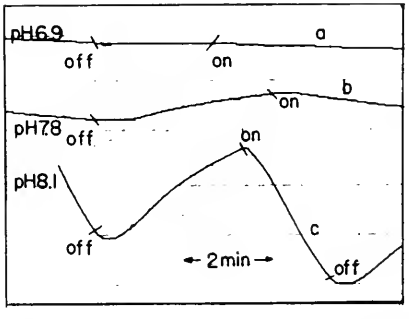

Fig. 1. Absence of acidity changes not attributable to carbon dioxide when light is turned on or off. Curves a and b: *Scenedesmus* in 0.002 *M* phosphate buffers nearly free of CO_2. Curve c control in 0.002 *M* bicarbonate.

trace means that carbon dioxide is neither released nor absorbed, or that both processes balance exactly.

What happens behind the cell wall within the cell is another matter. Obviously the method cannot distinguish between the decomposition of intracellular carbonates and true decarboxylations.

Many years ago I came to the conclusion that gross transients lasting for a minute or more are due to the interference of general metabolic reactions or their products with the normal course of photosynthesis, and that such phenomena do not reflect simply the sequence of incipient reactions building up the conditions in the steady state, for their cause evidently does not reside in the photosynthetic mechanism proper (6). In the main this has been borne out by the development in the intervening years. For instance, after a short anaerobic incubation, when respiratory and fermentative reactions are at

a minimum, photosynthesis starts and stops within a second and reaches the steady state a few seconds later (Fig. 6 and Fig. 3 in ref. 3).

The most talked about abnormal induction effect for carbon dioxide is perhaps the one found by Emerson and Lewis (7) in *Chlorella:* the *carbon dioxide gush* when the light is turned on. Our version with *Chlorella* at 15°C. is shown in Fig. 2. The upper curve shows the actual recording, the lower curve the corresponding computed rates.

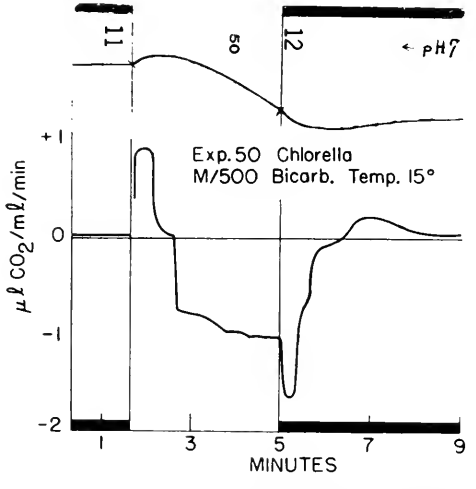

Fig. 2. Initial carbon dioxide evolution preceding its absorption at start of illumination in *Chlorella.* Strong "Pick-up" when light is turned off. Compare Emerson and Lewis (7, Fig. 4).

We have no machine to translate the upper into the lower curve. We merely take the tangents at a certain number of points—a simple, inaccurate, yet so far adequate way to see what is going on. In most cases the raw recording of pH changes already reveals the story. Figure 2 should be compared with the idealized drawing in the paper by Emerson and Lewis (7, Fig. 4) based on measurements with algae suspended in an acid medium. Important is the clear break toward a faster uptake of carbon dioxide the moment after darkening. This example may serve to demonstrate how our apparatus is capable of confirming faithfully the occurrence of such complex reactions which were found before with more sluggish methods.

The next observation I wish to discuss is seen in Fig. 3. The two heavy lines are 160 seconds apart. The light is turned on at the heavy line on the left, and off at the one on the right. The lower curve tells us how in a matter of a second or two the direction of the metabolic gas exchange may change upon illumination. The upper curve contains the feature that I find interesting. Here the first effect of light is to compensate respiration only. This *compensation period* lasts 20 to 40 sec-

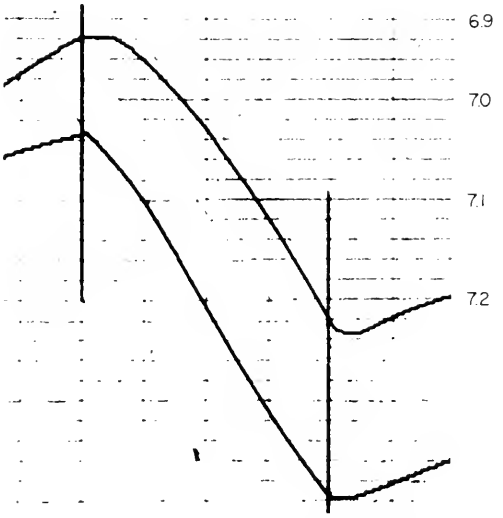

Fig. 3. Recording of induction periods in *Chlorella* under different conditions after repeated short dark and light periods. Length of light period between the two vertical lines: 160 seconds. Upper curve: 0.7% cell volume in 0.002 *M* bicarbonate between pH 6.4 and 6.9. Two-day culture in Warburg medium. Lower curve: 0.9 cell volume in 0.004 *M* bicarbonate between pH 7.5 and 7.9. Four-day culture in 0.05 *M* bicarbonate medium.

onds—in other cases even longer—and only after that time does photosynthesis start in earnest. This compensation effect is not a rare occurrence and not restricted to *Chlorella*. It was found also with *Scenedesmus* and *Stichococcus*. In acid media it seems to be the rule. It can be made to disappear when the algae have been grown in bicarbonate instead of in acid phosphate buffer, as was the case with the *Chlorella* producing the lower curve in Fig. 3. It may not be superfluous to emphasize that these observations have been made innumerable times during the past four years.

Such compensation effects are particularly extensive after long dark periods but tend to become shorter in repeated sequences of light and dark. In Fig. 4 the first of the compensation effects is always longer than the second. What completely erroneous results one would obtain

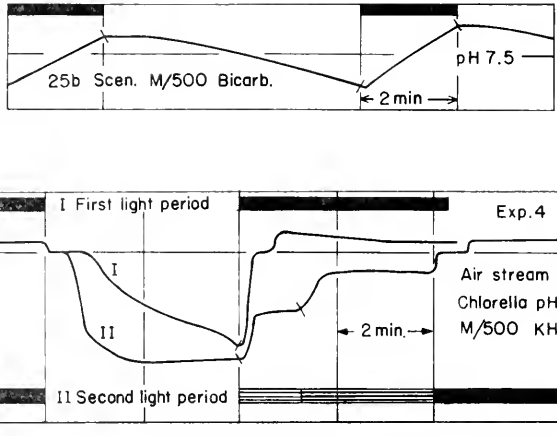

Fig. 4. Dependence of length of compensation period on length of preceding aerobic dark time. Upper curve: Recording showing a long compensation period after 2 hours dark time, a short one after only 2 minutes dark time. Low rate of photosynthesis. Lower curves: Rates of carbon dioxide uptake during the first illumination period after several hours of darkness (I), and during the second, following a dark time of about 4 minutes (II). In I the compensation lasts 1 minute the entire induction period over 4 minutes. In II the compensation is shortened to 25 seconds, the induction period to 90 seconds.

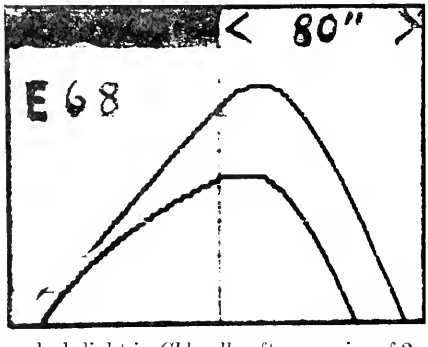

Fig. 5. Transitions dark-light in *Chlorella* after a series of 2-minute intermittencies; 0.6% cell volume in 0.002 *M* bicarbonate. Upper curve at pH 8.5, lower curve at pH 7.7.

if corresponding manometric pressure changes were believed to be caused by normal photosynthesis is obvious.

The exact flatness of the part of the curve immediately after the light has been turned on does not seem to have been caused by a fortuitous balance of the release and uptake of carbon dioxide aided by a lag in the response of the apparatus. Off hand, one would expect *a gradual change from respiration to photosynthesis.* Figure 5 shows this type of transition found with the same suspension of algae which

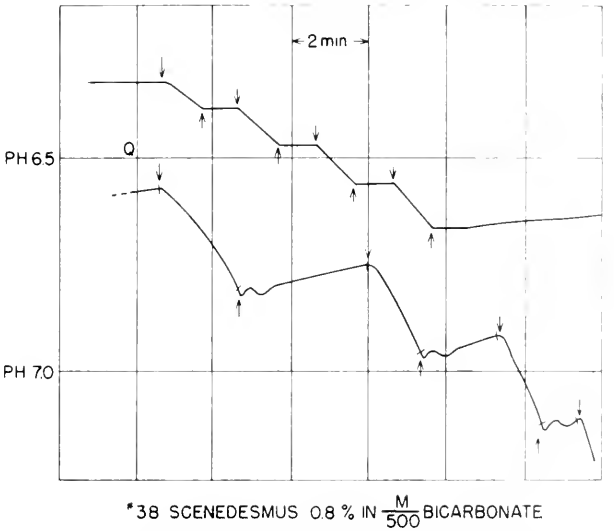

Fig. 6. Absence of conspicuous transients under anaerobic conditions and their reappearance with the beginning of respiration. Recording of pH changes during a succession of light (↓) and dark (↑) periods. The lower curve is the continuation of the upper one. *Scenedesmus*, 0.8% cell volume in 0.002 *M* bicarbonate, kept 30 minutes under hydrogen in the dark.

a short moment ago gave a flat compensation. In both these curves the rate of the response of the apparatus to the pH change in the dark and in the light is about the same, yet we have this striking difference in the shape of the transition during the first 30 seconds in the light. It is therefore the conditions in the algae and not that of the apparatus which determine the shape of the induction curve.

A gradual transition from carbon dioxide release to carbon dioxide absorption speaks for an independent respiration on which a slowly

starting photosynthesis is being superimposed. The sharp kink encountered in the majority of cases, particularly if followed by long flat compensation periods, forces us to assume a strong direct interference of the photochemical process with the respiratory metabolism. We should not forget that what we see here is related only to the carbon dioxide side of the picture, but there is no reason for assuming that the absorption of oxygen should become blocked in the light, as is the case with certain purple bacteria. Our experimental conditions are similar enough to those maintained in Brackett and Olson's experiments and in those of A. Brown to be quite sure that our algae

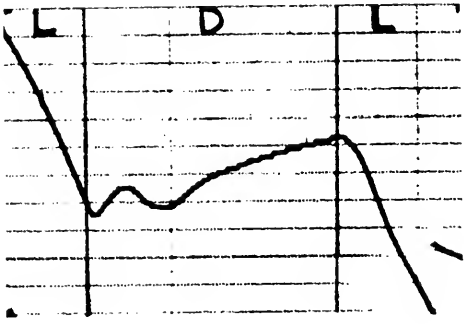

Fig. 7. Enlarged part of recording of Fig. 6 at pH 6.8. Dark time between heavy vertical lines 2 minutes. Transients after "light off" last about 1 minute, are probably caused by three superimposed reactions: (*1*) "Pick up" = $C_5 + C_1$; (*2*) "back reaction" = decarboxylation of dicarboxylic acid; (*3*) normal respiration.

continue to take up respiratory oxygen in the light. It is the release of respiratory carbon dioxide which is stopped or exactly compensated the moment the light strikes the cells. And occasionally this compensation reaction takes precedence over the normal photosynthetic one for times lasting up to a minute or even more. In other words respiratory intermediates are being reduced while the Benson-Calvin cycle is still inactive.

A corresponding effect is nearly always found when the light is turned off. After the carboxylation reaction—which is known to continue for a few seconds in the dark—has subsided, one sees a flat portion in the curve, indicating that respiration is not yet leading to a net release of carbon dioxide; in other words an oxidation of reduced

intermediates takes for a while precedence over the oxidative decarboxylations.

A very simple proof for the causal *connection of these transients with the oxidative metabolism* can be given. When air is replaced by nitrogen or hydrogen these transients disappear until enough photosynthetic oxygen has been produced to reverse at least part of the anaerobic

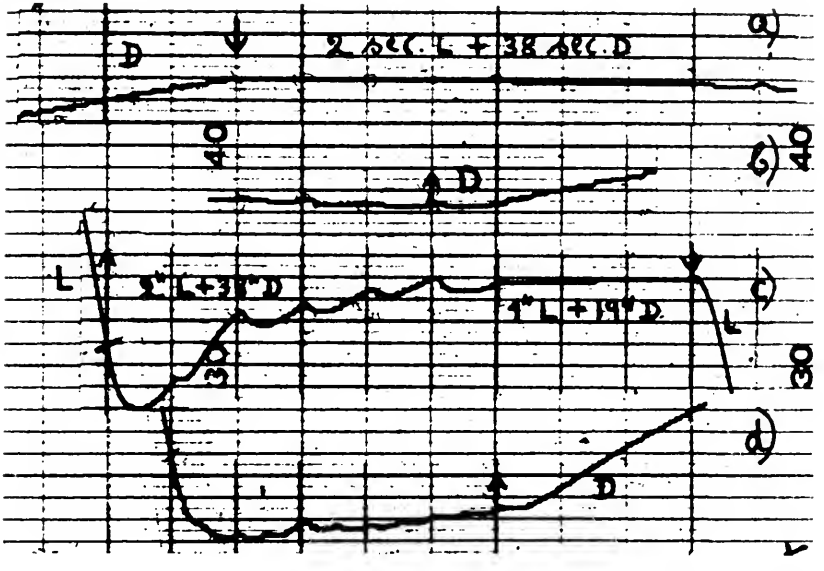

Fig. 8. Prolonged compensation of respiration brought about by 2 seconds of light given every 40 seconds. *Chlorella*, 0.9% cell volume in 0.002 *M* bicarbonate, pH 7.3. The curves *a*, *b*, *c*, *d* are consecutive parts of the same recording. See text.

conditions. Figure 6 (in which the lower curve is the direct continuation of the upper one) shows how a succession of dark and light periods (the light being rather strong) gives nothing but straightforward photosynthesis without conspicuous transients as long as the algae remain under anaerobic reducing conditions in hydrogen. But the eventual appearance of oxygen causes a most peculiar sequence of aftereffects in the dark, which can be seen enlarged in Fig. 7.

The question arises whether compensation reactions occur only when they last long enough to be seen so easily or whether they always are present at the start of photosynthesis in the light and must be

looked upon as *a normal component of all aerobic induction periods.* I believe Fig. 8 provides the answer.

These algae happen to start actual photosynthesis very quickly when the light is turned on—as, for instance, at the end of line *c*. In Fig. 8, *a*, *b*, *c*, and *d* are parts of a continuous recording. They were cut out of the chart and pasted below one another for easier comparison. If after a dark period the light is not left on for longer than 1 or 2 seconds, we obtain an immediate break in the respiration curve—but no photosynthesis.

Instead respiration remains compensated during the following 20 to 40 seconds in the dark (as after ↑ in line *b*) before it resumes its normal course. It is not difficult to find the right interval when a renewed flash of light will prevent the reappearance of respiration and prolong the compensation for another 40-second dark period. Beginning with ↓ on line *a* this sort of play with properly timed flashes produced a compensation period lasting here 8 minutes. Obviously the size of pools of intermediates and the reaction rates determine the outcome of such an experiment, and it is not too surprising to see how an interposed illumination lasting 40 seconds (between lines *b* and *c*) disturbs conditions. Now (line *c*) 2 seconds of light not only start a compensation reaction but also some photosynthesis followed by an enhanced respiration long before the 40 seconds of the former compensation period are over. By cutting the light as well as the dark period in half—leaving the integrated times unchanged—that is, giving now a 1-second flash every 20 seconds—restores the original compensation effect. Another minute of strong photosynthesis, between lines *c* and *d*, interferes with this arrangement. One second of light produces a compensation period in the dark which ends short of 19 seconds. We see that respiration starts each time just before the next flash initiates a new compensation. The last flash in this picture was given at ↑ line *d*, and following the dark pause of 18 seconds the carbon dioxide of respiration suddenly appears at a rate which now is three times that at the beginning of the experiment.

What do these recorded curves for carbon dioxide tell us about the nature of induction periods and aftereffects? The last-described effects make it clear that compensation reactions may occur even if they are not so obviously visible as in Fig. 3, and that they precede those reactions which lead somewhat later to the net uptake of carbon dioxide. Substances which in the dark usually decompose with the

evolution of carbon dioxide are evidently reduced in 1 or 2 seconds of strong light and their reoxidation to the previous level takes a comparatively long time. The fact that this first action of light does not lead automatically to a subsequent uptake of carbon dioxide may be explained in two ways. Either the substances reduced immediately at "light on" are not part of the photosynthetic carbon cycle or another action of light is needed to move the precursor of the carbon dioxide acceptor all the way around the cycle until carbon dioxide is picked up to give new PGA, and this second reaction does not start simultaneously with the first. Removing, for instance, respiratory PGA by reduction to triose, will certainly prevent it from going into pyruvate, etc. Now if more light is needed to drive the phosphorylation in the carboxylation cycle and the light flash was too short, this triose will simply be reoxidized.

Any deviation in the concentration of PGA from that prevailing in the stationary light phase, as well as variations in the rate of photosynthetic phosphorylation, may well produce transients similar to those observed. Furthermore, there is evidence for the appearance of other kinds of reductive carboxylations in the light (8). The instantaneous nature of the break in the evolution of respiratory carbon dioxide when the light is turned on calls, however, for a more specific explanation. It can best be understood if the decarboxylation steps in the respiratory cycle (Krebs cycle) were blocked. This would happen if the pyridine nucleotides taking part as coenzymes became reduced.

Weigl and Calvin (9) showed that light prevents the entrance of freshly assimilated carbon into the Krebs cycle. And Calvin (10) later proposed a special "valve" action—assigned to thioctic acid—to stop the decarboxylation of pyruvate. Plausible as Calvin's hypothesis seems, it is not so serviceable for our purposes as the more general assumption of the reduction of pyridine nucleotides. According to Brown and Good (11), this reduction best explains the compensation by light of respiration in cyanide-poisoned *Chlorella*. There is no doubt that a reduction of pyridine nucleotides will occur when conditions are right.

Finally, we have to see whether such an explanation for our carbon dioxide transients remains valid in view of Brackett and Olson's observations on oxygen transients (see this book, pages 412–418). I believe the explanation also holds there without any straining or ad hoc

assumptions. They themselves have pointed out that the appearance and shape of the oxygen gushes depend on the oxidative metabolism in the dark but are independent of carbon dioxide concentration. Transferring our explanation of carbon dioxide transients to the oxygen side we arrive at the following picture: The first sharp oxygen spike occurs when the coenzymes, and consequently some intermediates, are being reduced. This spike may stand alone whenever the photosynthetic carbon cycle is inhibited either for lack of carbon dioxide or other reasons. The spike may disappear under strictly anaerobic conditions, the coenzymes then being present already in their reduced states and oxidized intermediates practically absent. The dip in the rate of oxygen evolution following the first gush—an observation made also by other investigators ((12), and Fig. 8 in ref. 3)—is brought about by two or three superimposed reactions: a momentary lack of hydrogen acceptors, a predominance of photosynthetic phosphorylations needed to promote the carbon cycle, and perhaps an increased rate of reoxidation. The third reaction should reveal itself as an increase in the rate of respiration whenever the latter was abnormally low ("starved algae") at the start of the experiment.

The aftereffects, i.e., the transients which we as well as Brackett and Olson have seen when the light is turned off, are to be expected when the reactions responsible for the induction are being reversed. In comparing the slopes of the recorded curves at "on" and "off" we have to keep in mind, however, that photosynthesis has in the meantime built up pools of intermediates which were not there before—giving rise to the continued "pick up" of carbon dioxide, for instance—and that the overall rate of respiration is generally slower than that of the light-driven reactions.

Discussion

Frenkel: If you speak of phosphorylation you have to consider that this might be accompanied by pH changes.

Gaffron: But these are inside the cells.

Frenkel: But the pH inside the cells is likely to change. For instance, in the case of a phosphorylation reaction, by phosphate uptake.

Gaffron: You have to take all the different possibilities into account. The explanation has to fit Olson and Brackett's experiment. It has to fit the Calvin cycle.

Brown: This is a specific case of a general statement, that if acid of any kind, other than carbon dioxide, is produced or consumed, the pH will change.

Gaffron: Precisely.

Frenkel: You also have to consider that phosphorylation by itself will produce chemical changes.

Gaffron: This depends at what pH you have carbonates in the cell which decompose in order to give carbon dioxide.

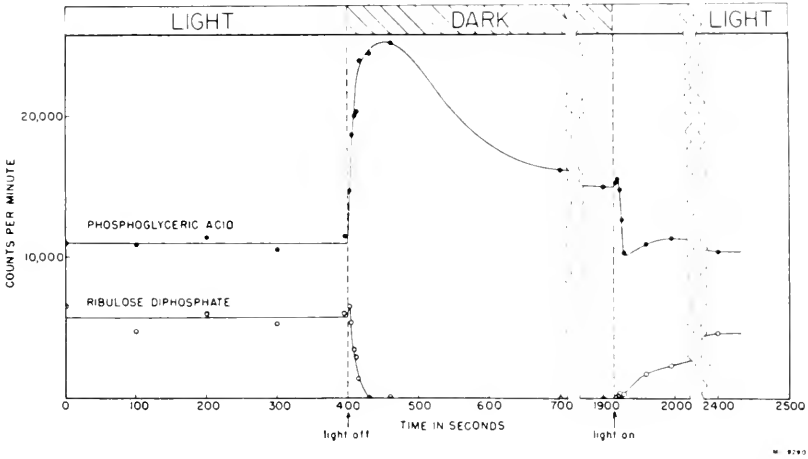

Fig. 9. Light-dark changes in concentrations of PGA and RuDP (after Bassham, Shibata, and Steenberg).

Bassham: I have some data on the specific carbon compound that relate very closely to what Dr. Gaffron has been talking about. Unfortunately, I did not foresee that they would be as important in this context as they are. Just some curves on specific compounds that explain these results. Figure 9 shows two curves which might explain these results. I won't explain the system except to say that it is doing steady state photosynthesis with constant specific activity. The light and dark periods are marked. The upper curve shows changes in the PGA level. On turning off the light, it comes up as shown to a new high level. Turning the light on again makes it go down first, then return to its initial level in the light. The lower curve is ribulose diphosphate. On turning the light off, its level falls rapidly after a momentary small rise, and then it practically disappears. In the light again, the level of ribulose diphosphate starts to rise but rather slowly.

What does this mean? It simply means that in the light we have a steady-state concentration of PGA. On turning off the light the PGA continues to be formed by the carboxylation of the ribulose diphosphate. We get two molecules of PGA for each ribulose used up. The initial slope of this curve is twice the rate of entry of CO_2 into the photosynthetic cycle, proving that you do get two molecules of PGA per carbon dioxide going in. The increase in the citric acid cycle intermediates

is significant because it means that in the chloroplasts as soon as you turn the light off the photosynthetic intermediates are converted into Krebs cycle intermediates, and on turning the light on again, some of them are converted back over to photosynthetic intermediates.

Gaffron: This is very agreeable. We can explain nearly everything. A question, of course, concerns the quantities we have. We also ought to be careful in comparing the times.

Bassham: I think they are in perfect agreement with all your results.

Blinks: I thought I saw one place where there might have been a flash of CO_2 gas. You meant the Emerson CO_2 effect?

Gaffron: Yes. I have to use *Chorella* and keep them cool. Then I get it, but it is a long-drawn-out affair.

Wassink: I would like to comment that Dr. Gaffron probably recalls that we have done exactly the same thing. With a suitable sequence of light and dark periods, you can keep fluorescence at a higher level, and you can get photosynthesis during that situation and find certain characteristics, which I don't recall. However, these are different from those in the steady-state illumination and may have a bearing on these compounds and still other ones that are engaged in the final phases of photosynthesis.

Gaffron: Observations of fluorescence and chemiluminescence first have to be taken at their face value from the point of view of the physicists and their interpretation sought simply in terms of what the pigments themselves can do when illuminated. This is difficult enough. Following this, we have to collect biochemical data on the kinetics of intermediates. A joint physical and biochemical approach may permit a satisfactory explanation of induction periods.

Kok: I want to point out that to me it appears premature to hypothetically postulate biochemical pathways for explaining these complicated effects. I could have shown all sorts of anomalous oxygen and carbon dioxide exchanges, which we measured simultaneously under a variety of conditions. One can obtain just about any result.

Gaffron: Therefore we should concentrate on those which are typical and can easily be reproduced. These are a selected few, which make sense.

Brown: Have you done any measurements at low pH where you do not have a good bicarbonate buffer?

Gaffron: Yes, the only difference I found is that, unfortunately, one cannot calculate the rates. For pure bicarbonate buffers we can calculate nicely, as Dr. Rosenberg has shown, and we can convert these wiggles into quantitative rates.

Brown: I mean well out of the buffer range.

Gaffron: I can wash the algae three times with distilled water, saturate with 5% CO_2, and watch what happens there. It turns out that the general shape of the curve remains identical. So we may consider ourselves on safe ground.

Brown: I don't want distilled water because you still have a buffer to some extent probably. I want a dilute buffer, at low pH. Do you have that kind of data?

Gaffron: For a dilute carbonate-free phosphate buffer near neutrality see Fig. 1. I have no experiments with buffers in the truly acid range.

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Chromatic Transients in Photosynthesis of Red Algae

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Induction lags, oxygen "spikes," carbon dioxide gushes, and other transient phenomena are well known in the first moments of photosynthesis, being especially marked after long dark periods, under anoxic conditions, or in the presence of high CO_2 concentrations. They are much less conspicuous, though also present, under alterations of light intensity, but seem to have been little investigated when the wavelength of illumination is altered (under constant intensity or effectiveness). The latter situation has been studied in several marine red algae, yielding time courses which may be designated "chromatic transients."

The method was essentially that of Blinks and Skow (1) using the polarographic or amperometric oxygen cathode (stationary platinum electrode polarized at 0.5 volt). The tissue was held in direct contact with the electrode by a strip of permeable cellophane (*cf.* Haxo and Blinks (3)). Measurement was by Leeds and Northrup "Speedomax," which recorded the potential across a decade resistance inserted in the polarographic circuit. Oxygen diffuses across the tissue from the surrounding sea water (which can be aerated and/or flowing) setting up a steady current flow, after initial polarization has occurred. This is the base line in the dark, representing the respiration of the tissue by the distance below compensation point (dashed line in Figs. 1 and 2). On illumination (by grating monochromator) a new steady state is attained (2) (Fig. 1), owing to the diffusion of photosynthetically produced oxygen to the electrode. Since the tissue is usually but one cell thick, diffusion is only across the cell wall, and the response is very rapid, a new steady state being generally attained in less than a minute.

Under favorable circumstances (active photosynthesis in very thin thalli) a difference in time course with red algae can be observed between illuminations with red and with green light. An example is shown in Fig. 1. The initial rate tends to be higher in green light, lead-

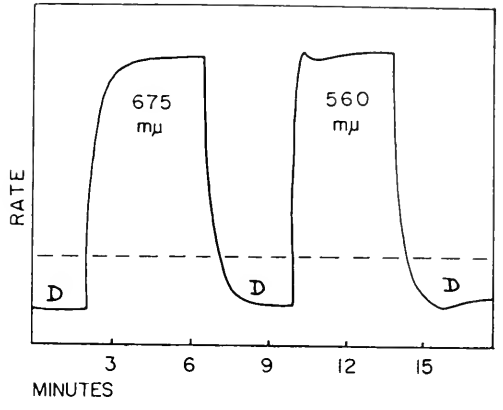

Fig. 1. Time course of oxygen evolution by *Porphyra perforata*, first in red light (675 mμ); then in green (560 mμ), of intensity adjusted to give the same steady-state rates. Dark exposures (D) are shown before, between, and after the light exposures. Dotted line indicates compensation point. Time in minutes. Some records show no cusp in green light, but a more rapid rise to the final rate. (Tracing of "Speedomax" recording from oxygen cathode circuit.) Note the somewhat enhanced respiration after green light.

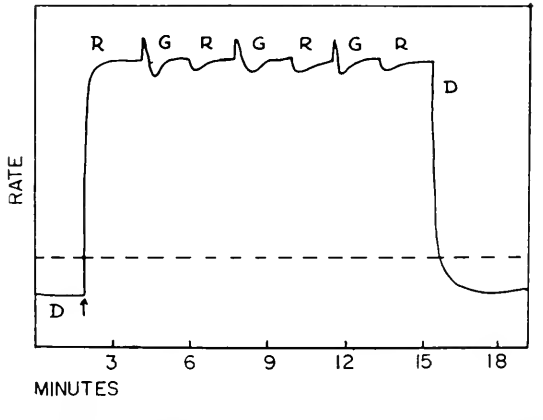

Fig. 2. Time course of photosynthetic time course in *P. perforata* exposed to alternating red light (R) and green light (G) of equal steady state effectiveness. (R = 675 mμ; G = 540 mμ.) Short oxygen "gushes" occur at each exposure to green light, followed by a depression and recovery; in red light the level at first falls, then recovers. Dark periods (D) before and after the light sequence; compensation point shown by the dotted line.

ing to more rapid attainment of the steady state, or even with a cusp slightly above the final value, while in red light the steady level is attained more slowly. These differences are not always evident, especially the cusp, which in thicker tissues seems to be swamped out in diffusion.

The transients become more striking when alternate exposures are given to red and to green light without intervening dark periods. Figure 2 is a tracing of a Speedomax recording, showing first the dark

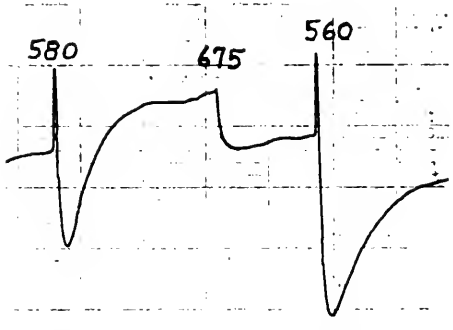

Fig. 3. Actual Speedomax recording of the transients on changing from red to yellow or green light. The initial trace is the rate in red light (675 mμ); on exposure to yellow light (580 mμ) there is a very sharp oxygen gush, followed by an equal depression of considerably greater length, with final recovery to a somewhat higher level than in the red, which follows (675 mμ). The gush and depression are even sharper in the green (560 mμ). These transients represent some 15% or 20% of the total photosynthesis, the base line lying well below the bottom of this record. Vertical lines, 100 seconds apart.

value, then the rather regular attainment of a steady photosynthetic rate in red light (675 mμ, well above compensation but far from saturation). On shifting the monochromator setting instantly to green light (540 mμ) there is an immediate cusp (oxygen gush) followed by a longer depression, with recovery in about a minute to the stationary state. On going again back to the red light (675 mμ) a slight depression is observed, followed by recovery. These are repeated any number of times, with little change in the characteristic courses, providing each has gone to completion. (Alternations which are too short show smaller cusps and depressions.) On darkening, the respiration value is quickly attained (D).

Figures 3 and 4 are actual photographs of Speedomax records, showing the extremely sharp initial cusp in green, yellow or orange light and the very large depression immediately following. This is especially marked at 560 mμ (Fig. 3). There is never a cusp in red light—only the depression followed by recovery. The characteristic time courses are found only when the wavelength of illumination is changed, not the intensity. They are not dependent upon exactly equal steady states; the several components are present (Fig. 4) at 600 mμ at a higher steady state, or at 560 (lower than red light). They can also be evoked on going from green to yellow light, or vice versa (between the absorption regions of phycoerythrin and phycocyanin). They seem indeed to be characteristic of the phycobilin

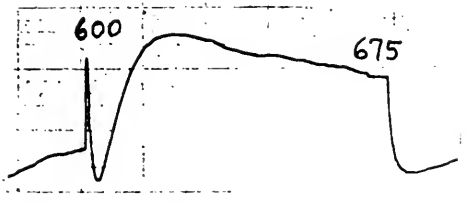

Fig. 4. Speedomax record of photosynthetic transient on changing wavelength from red (675 mμ) to orange (600 mμ) and back to red (675 mμ) of equal intensity. Time marks 100 seconds. Base line well below the record.

pigments: no trace of these transients has been seen in *green* or *brown* algae on alternating light exposures between chlorophyll and carotenoid absorption regions.

The explanation for these transients is not fully apparent. They are not large compared to some of the effects following long darkness, amounting only to some 10% or 15% of the total oxygen evolution level. They are, however, remarkably consistent and reproducible amongst the algae well-adapted to show them (monostromatous forms with a minimum of diffusion distance to electrode). They have been most conspicuous in three species of *Porphyra*. Higher red algae—even where monostromatous—do not show them as well. Since *Porphyra* is the genus in which activation of chlorophyll by red light is also possible (4), it may be that the same mechanism is involved. Thus even during a short exposure to red light there is partial recovery after the lowered initial rate; and conversely an initially higher rate in green light (due to this recovery?) is followed by a

rapid fall—possibly due to inactivation of some of the chlorophyll, to which energy must be transferred from phycoerythrin. Studies of fluorescence during this transient phase would be desirable and will be attempted.

Another possibility is an altered respiratory rate during the first moments of light absorption by phycoerythrin. Only mass spectrographic studies could determine this, and it seems likely that their time resolution might not be adequate to follow these essentially second-order effects.

It is clear, however, that the transition from chlorophyll absorption to phycoerythrin (or phycocyanin) absorption is not completely smooth, even though the final photosynthetic rate may be the same. There is a "grinding of the gears" at the moment of change. When this is understood, there may emerge a better understanding of the remarkable "inactive chlorophyll" of the red algae.

Discussion

Wassink: Is there a dark period between the periods of illumination at 600 and 675 mμ?

Blinks: No, there is no dark period. We are simply going from one wavelength to another.

Hendley: Would the plants see these two colors of light at the same intensity?

Blinks: They were in many cases the same intensity. They can, however, be so adjusted that the steady-state photosynthesis is the same instead. The transients occur in either case.

Gaffron: Changes in light intensity cause transition effects and they are also one-sided. I have not seen them, from low light to high light. However, from higher light back to low light there is always a rather long disturbance.

Blinks: It makes no difference if the intensities are alike or not, you still have the transients. If you adjust intensity so that photosynthesis is *equal in the steady state* you get them best of all.

Brown: You say you do not see these in green algae?

Blinks: No, not in going from the carotenoid to chlorophyll absorption regions or vice versa.

Strehler: Does it happen at very high light intensities, that is, at saturation intensities?

Blinks: No, these are at intermediate intensities.

Emerson: We have been doing somewhat similar experiments with red and green algae, looking at the subsequent respiration after exposure to different wavelengths of light. In the case of red algae there is a strong promoter effect of green and blue-green light on the subsequent respiration, analogous to the depressing effect on photosynthesis described by Emerson and Lewis for *Chlorella* at about 480 mμ in the blue.

Blinks: I think quite possibly these are "*solarization*" effects on a very small scale, but respiration may be involved.

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Transients in Acid Production by Purple Sulfur Bacteria

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A study of pH changes in suspensions of a photosynthetic bacterium, *Chromatium*, strain D, has revealed unexpected variations in the rates of acid production and consumption (CO_2 or other acid) during alternate dark and light periods.

The pH was measured in a static, single-phase liquid system with glass and calomel electrodes, Beckman model R pH meter and recorder—a technique which has been used previously in this laboratory (1–2). pH changes in suspensions of the bacteria in phosphate or bicarbonate buffers were recorded with a lag of less than 0.8 second at 25°C. It is the low inertia of the method which is its chief virtue.

Some of the transient effects which may be observed are illustrated in Fig. 1, which is a record of pH changes in an anaerobic suspension of *Chromatium* in dilute bicarbonate buffer during a sequence of dark-light-dark periods. In the region labeled "*F*" there is a steady rate of acidification due to endogenous fermentation. At the arrow the suspension was illuminated and an immediate "acid gush" occurred, labeled "*A*." (Disregard for the moment the dashed lines.) The "acid gush" was quickly followed by an uptake of acid at "*R*" clearly distinct from steady-state photoreduction of CO_2, "*P*."

When the light was turned off, at the second arrow, a rapid "dark gush" of acid occurred, indicated at "*D*." Following this there was a secondary uptake of acid at "*U*" before a steady rate of endogenous fermentation was reached.

The dashed lines indicate characteristic pH changes which might have taken place had the conditions been somewhat different. The acid gush is dependent on a rather long preceding dark period; and, had the dark period prior to the first illumination in Fig. 1 been shorter than about $^1/_2$ minute, the acid gush would probably have been replaced by a rapid "initial uptake" of acid, indicated at "*I*." Fre-

quently, after very long dark periods, a secondary acid evolution, "*S*," may follow the acid gush.

The general pattern of pH changes shown in Fig. 1 has been observed hundreds of times, but it must be emphasized that the rates and magnitudes of the effects shown vary widely in an as yet unpredictable fashion from culture to culture and even in the same suspension while it is under observation.

That the anomalous pH changes are not artifacts of instrumentation but are related to photoreduction is shown by the fact that heat treatments and certain inhibitors which inhibit photoreduction also abolish all pH changes caused by light.

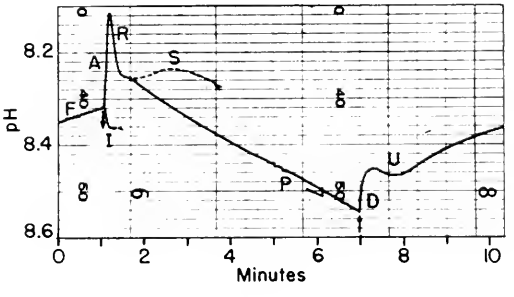

Fig. 1.

Since stirring of the suspension was found not to influence the results, it was assumed until recently that the suspension remained homogeneous with respect to pH. It has been found, however, that the tendency of the bacteria to form a film on the surface of the glass electrode causes the pH changes in this surface layer to exceed those in the rest of the suspension. For rapid pH changes this inhomogeneity may be quite marked even though the film may be almost invisible, and despite stirring of the solution.

The build-up of the film may be prevented by frequently cleaning the electrode. It is possible to correct for it by a separate measurement of the pH changes due to the film of bacteria alone in plain buffer after draining the bacterial suspension. A number of new measurements have recently been made of the acid gush and initial uptake where the film effect was kept negligibly small. These new values have been substituted in this manuscript for the erroneously large values reported at the Gatlinburg meeting.

Space permits a discussion of only two of the induction effects illustrated in Fig. 1, the acid gush and the initial uptake. Effects similar to these have been studied in green plants and I shall try to make a brief comparison.

The acid gush. Blinks and Skow (3) were the first to observe an initial pH decrease upon illumination in suspensions of the alga *Stephanoptera* and at the surface of leaves of higher plants. Emerson and Lewis (4) later published a detailed manometric study of an initial "CO_2 gush" from suspensions of *Chlorella* in acid phosphate solution.

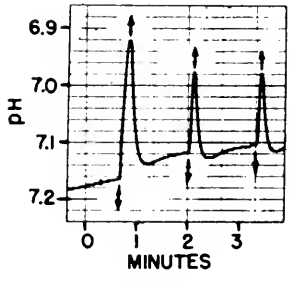

Fig. 2.

Like the acid gush of the bacteria, the CO_2 gush described by Emerson and Lewis is, up to a point, larger the longer the dark period preceding the illumination. After long dark periods both gushes may reach the equivalent of 0.2 volume of CO_2 per volume of cells.

The bacterial gush is usually followed in the light by a roughly equivalent uptake of acid ("*R*" in Fig. 1) clearly distinct from steady-state photoreduction. This reversal of the acid gush in the light has not been reported for the gush in algae, where the reaction is either absent or so sluggish that it cannot be distinguished from photosynthesis.

On the other hand, both the bacterial and algal gushes are reversed in the dark if the light is turned off during the gush. This may be seen in Fig. 2, which shows the pH changes in a bacterial suspension which was illuminated three times, the light being turned off when the acid gush reached its maximum deflection. Each time the pH was rapidly restored in the dark. The acid gush and the immediately

following acid uptake seem to be closely related processes. Any explanation of the former should also account for the latter.

The quantum yield of the gush appears to be high both in algae and in bacteria. The measurements of Emerson and Lewis (4) indicate a quantum yield of 0.8 or higher. Although the absolute quantum yield of the bacterial acid gush has not been measured, it has been found that at rate-limiting light intensities the rate of CO_2 (or other acid) production for the acid gush is four to six times higher than the rate of CO_2 reduction with H_2 as H-donor. This indicates that the maximum quantum yield (CO_2 or H^+ produced/quantum) of the acid gush is probably in the neighborhood of $^1/_2$, since most measurements of the maximum quantum yield for photoreduction indicate values of $^1/_{10}$ to $^1/_8$.

The CO_2 gush studied by Emerson and Lewis in algae shows a dependence on oxygen which is not shared by the acid gush of *Chromatium*, which has normally been examined under the same strictly anaerobic conditions which are required for growth of this organism. The effect of oxygen is probably not specific for the CO_2 gush, since it is well known that photosynthesis in *Chlorella* is also markedly inhibited by prolonged anaerobiosis under the acid conditions obtaining in the experiments of Emerson and Lewis.

The CO_2 gush in algae requires a rather high partial pressure of CO_2—a fact which suggested to Emerson and Lewis (4) and Franck (5) that some sort of reversible carboxylation is involved. If the P (CO_2) is lowered to 0.5% the CO_2 gush falls to a negligible value. The acid gush in *Chromatium*, on the other hand, does not seem to be similarly affected by the CO_2 pressure since it still is of a significant size at a CO_2 pressure which is so low (less than 0.02%) that photoreduction is reduced to compensation of fermentation reactions.

The data on hand do not rule out the possibility that the gush both in bacteria and in algae is caused by the production of an acid other than CO_2 which displaces CO_2 from a bicarbonate reservoir in the cell. This would presuppose an intracellular bicarbonate concentration, under some conditions, of at least 0.009 M to explain the largest acid gushes which have been observed. It may prove possible to test this hypothesis by a direct measurement of intracellular bicarbonate levels.

The experiments of Emerson and Lewis (4) exclude the possibility that the CO_2 gush in *Chlorella* is due to the excretion of a strong acid

from the cell. This remains a possibility, however, for the bacterial acid gush, since our measurements were restricted to a pH above 6 owing to the sensitivity of the bacteria to acid conditions.

That the acid gush may attain the equivalent of 0.2 volume of carbon dioxide suggests, under those conditions, an intermediate change in intracellular concentration to the extent of 0.009 *N*. If the intermediate were stable, such a concentration change should be detectable by the tracer technique used by Calvin and Massini (6) for measuring pool sizes of intermediates. We are at present making a quantitative comparison of C^{14}-labeled intermediate pool changes during the acid gush with the pH changes in the suspension being measured simultaneously.

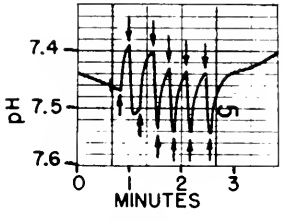

Fig. 3.

The initial uptake. When short dark periods follow a longer period of photoreduction in H_2 an initial increase in pH rather than an acid gush usually occurs on reillumination, clearly distinguished from steady-state photoreduction by its greater slope. The rate of acid uptake at low light intensity is approximately two times higher than photoreduction with H_2 as H-donor. In magnitude the initial uptake may exceed the equivalent of 0.05 volume of CO_2 per volume of cells, suggesting a change in the intracellular concentration of some intermediate of about 0.002 *N*.

The initial uptake is followed in the dark by a reverse reaction of approximately equal size, the "dark gush." The connection between the initial uptake and dark gush is best shown in Fig. 3, which is a record of pH changes in a suspension which was subjected to a series of short light and dark periods. Initial uptakes followed by dark gushes of the same size have also been seen in heat-treated leaves by van der Veen (7). The quantum yield of the initial uptake in green plants has not been determined.

As in the case of the acid gush we are forced to consider not only carboxylation reactions as possible causes for the initial uptake and dark gush but also a multitude of other metabolic reactions which are known to produce or consume other acids besides CO_2. These might affect the external pH by a direct excretion of the acid involved (8) or, more likely, through changes in intracellular bicarbonate.

Among the reactions involved in the current picture of photosynthesis which might be expected to rapidly produce or take up acid to an extent sufficient to explain the initial uptake and dark gush, the most evident are those concerned with the formation and utilization of PGA and inorganic phosphate.

A reduction of the PGA pool largely to neutral triose before the concentration of the CO_2 acceptor has a chance to build up offers a possible explanation for the initial acid uptake. The dark gush might be the result of the rapid conversion in the dark of the ribulose diphosphate pool to PGA (6) resulting in the formation of two carboxyl groups for each CO_2 used. It should be noted that if there were very little intracellular bicarbonate, these internal acidity changes might not affect the external medium at all, in which case only one change would be observed: a CO_2 uptake on darkening due to the carboxylation of ribulose diphosphate.

While PGA pool changes may play a role, preliminary measurements of ATP levels during the initial uptake and dark gush suggest that changes in phosphate esterification may account for the bulk of the pH changes. We found that the formation of ATP, measured by the method of Strehler and Totter (9), during the first moments of illumination is of the same order of magnitude as the acid taken up in the initial uptake. During the dark gush the ATP falls to or below the level before illumination.

Further studies are required before the contribution of various factors to the initial uptake and dark gush can be quantitatively evaluated.

Discussion

Strehler: Interestingly, the amount of ATP in these bacteria is many times higher, perhaps 10 to 100 times higher, than what you find in the *Chlorella;* and, although there is only about a 50% change in the total ATP content that we detect here, it is quite an enormous change in comparison with what *Chlorella* does with the same amount of light.

Wassink: I was just wondering exactly what you meant by these quantum efficieneies. Have you any indieation that the meehanism is the same? Is any hydrogen taken out during these initial effects?

Hendley: No, I have no measurements of hydrogen changes.

Wassink: You, of course, are not sure that the comparison rates of CO_2 and hydrogen are the same during the initial effect?

Hendley: No, they may be different.

Wassink: That was observed in the case of initial effects that Emerson studied, and so on, in *Chlorella.*

Hendley: Yes.

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Part VI

FORMATION AND CONDITION OF CHLOROPHYLL IN THE LIVING CELL

Chloroplast Structure and Its Relation to Photosynthesis

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Considerable progress has been made in recent years in the study of the structures of the chloroplast. I should like to summarize very briefly what is known of these structures and discuss what they might represent.

On the basis of the electron microscope studies of various workers (1) the following interpretation is presented of the structure of the generalized chloroplast of higher plants. A chloroplast is a saucer-shaped structure 4 to 6 μ in diameter and 0.5 to 1 μ in thickness. The chloroplast is surrounded by a semipermeable membrane 50 to 100 A thick, that is, equivalent to the diameter of 1 or 2 protein molecules. Each chloroplast contains about 50 dense "grana" which lie embedded in a colorless stroma or protein matrix.

A dense granum has the shape of a column or cylinder 4000 to 6000 A in diameter and 5000 to 8000 A in height. Each granum consists of a stack of 15 or more dense parallel membranes or lamellae, piled one on top of another like a stack of poker chips. The thickness of a lamella is about 35 to 50 A. In one interpretation a pair of adjacent lamellae is considered to represent the upper and lower membrane of a "disc." The disc would be as wide as a granum (4000 to 6000 A) and have an overall thickness of 130 A; the disc would consist of a dense upper and lower membrane (each 35 A thick) enclosing a space of about 65 A between the membranes. Attached to each upper and lower membrane of a disc is a delicate membrane, 30 A thick, which extends out into the stroma and appears to connect one granum with another. In addition there is a space of about 65 A which separates these fine membranes from each other. Thus, proceeding downward through a granum we meet in succession a disc membrane 35 A thick, an intradisc space of 65 A, a disc membrane of 35 A, an interlamellar membrane of 30 A, a space of 65 A, an interlamellar membrane of 30 A, and again a disc membrane of 35 A, etc. (2).

The chloroplast of an alga like *Euglena* would appear to represent a single granum. Such a granum contains about 20 dense parallel membranes which extend the length and width of the chloroplast. These membranes are thicker and the interspaces are greater than those observed in the grana of higher plants.

When a *Euglena* organism is placed in the dark, it loses its chlorophyll; at the same time the membranes disappear from the chloroplast. On placing such a colorless *Euglena* in the light it gradually becomes green. At the same time the dense membranes appear (3). The greening thus appears to consist not only of a process of chlorophyll synthesis but concomitantly there must occur carotenoid and protein synthesis and the structural organization of these molecules.

The fine details that have been revealed by the electron microscope depend on a technique of tissue fixation in buffered OsO_4, subsequent dehydration through alcohols, embedding in a methacrylate polymer, and fine sectioning (4). Our studies of this procedure applied to chloroplasts suggest that the chlorophylls are leached out during the dehydration stages so that the final sections as viewed in the electron microscope may be assumed to be devoid of chlorophyll. We have also found that by this procedure carrot chromoplasts lose their carotenes.

The fixed insoluble membranes that are observed in thin sectioning of chloroplasts probably represent protein material. A photograph by Von Wettstein (5) suggests that a disc membrane may be made up of spherical protein molecules of about 35 A diameter. This would mean that the membrane would represent a monomolecular layer of protein.

Where might chlorophyll be localized in the chloroplast? There is sufficient evidence to indicate that chlorophyll is probably localized in the grana and not in the stroma. There is also evidence from studies with polarized light that chlorophyll is localized in layers within the grana. Let us assume that chlorophyll is localized within the disc, i.e., within the 65 A space between the upper and lower disc membrane. If the disc contained a monolayer of chlorophyll molecules with the plane of the porphyrin heads all lying flat and adjacent to each other in the monolayer, then there would be room for about 90,000 molecules per disc, and the concentration of chlorophyll inside the disc would be about 0.15 *M*. Other estimates suggest that if chlorophyll were localized here, then chlorophyll would take up about

one-fourth of the volume of this intradisc region. The high concentration of chlorophyll that may be estimated to exist in specific regions of the grana would be compatible with the hypothesis of a photosynthetic unit in which light energy could be transmitted through several hundred chlorophyll molecules before it was trapped in a "sink."

Is there any evidence for the specific orientation of chlorophyll molecules with respect to each other? Such orientations, if they occurred, might help to explain the great efficiency with which the energy of a photon could be transmitted through a photosynthetic unit. Goedheer (6) has concluded from studies of the birefringence of chloroplasts in the neighborhood of the chlorophyll band that there is a small amount of orientation, the porphyrin planes of the chlorophyll being oriented parallel to the disc membranes. However, Menke and Menke (7) question this interpretation. The very low yields of polarized fluorescent light, emitted by *Chlorella* when illuminated with incident polarized light, suggest that there is very little orientation of chlorophylls in a photosynthetic unit (8). However, on the basis of calculations of Arnold and Oppenheimer (9) it does not appear to be necessary to assume a rigid orientation of porphyrin planes stacked parallel to each other in order to account for energy transfer in the photosynthetic unit.

Sometimes when we know what a structure is we can explain what it does. Here, the knowledge of the structure of the chloroplast makes us aware of how very much we don't know of the mechanisms of photosynthesis.

Discussion

Latimer: There was mention of Goedheer's paper in connection with dichroism of chloroplasts. Goedheer also observed double refraction as did Dr. Menke later. Menke did this work in 1944 and just got around to publishing it. Double refraction and dichroism are essentially due to the same phenomenon, namely, to orientation of the molecules. If they were not oriented, a double refraction would not have been observed, but it was very clearly observed with the very interesting wavelength dependence which you saw, indicating some very definite organization (Latimer, this book, page 100). The degree of organization is hard to measure quantitatively but it showed up very nicely.

Granick: I believe Menke had a recent paper in *Z. Naturforsch., 10b,* 416 (1955) in which he revised and criticized Goedheer's work.

Rabinowitch: One word about double refraction. Of course, double refraction indicates that some molecules are arranged in a parallel way, and the suggestion

was that these might be lipoid molecules, perhaps, carotenoid molecules. However, it occurs to me that this double refraction showed the anomaly in the chlorophyll absorption band. Therefore, it must be the chlorophyll molecules which are arranged in some sort of regular pattern.

Those who were here a few years ago may remember Dr. Vatter, who showed some of his electron microscope slides. He has now made slides which are more beautiful than Steinman's, although Steinman perhaps has something much clearer. Vatter has pictures which show very clearly the development of structure in the course of evolution from chloroplast to the finished plastid. I strongly suggest that whoever is interested write to him and ask him for these pictures because they are so very much more beautiful than anything I have seen in the literature.

Unfortunately, I haven't yet studied his thesis enough to be able to summarize it properly.

Granick: The problem here is not whether the chlorophyll is localized in layers, but whether the chlorophylls are oriented with respect to each other within the layers. The presence of chlorophyll in the grana layers might explain dichroism, but does it explain necessarily whether the chlorophyll molecules are oriented with respect to each other? What might be needed to transmit light energy with great efficiency are chlorophyll molecules oriented with respect to each other.

Rabinowitch: There are two types of dichroism, morphic and intrinsic. I think what is in question here implies that the molecules are organized in parallel.

I was amazed to hear you say the chlorophyll is not in the fixed and sectioned chloroplasts which are examined in the electron microscope. Can you say why the chlorophyll is not there?

Granick: When chloroplasts are fixed by the customary OsO_4 procedure all the chlorophyll is leached out during dehydration in the alcohols.

Bassham: In view of the possible connection between dichroism and protein molecules I wonder whether Dr. Takashima would like to say something with regard to his protein chlorophyll crystals. The properties which you described sounded as though they might fit into that picture.

Takashima: The molecular weight was about 20,000.

Strehler: First, I wonder if you can tell me whether there is any chance that the membrane from the chloroplast is actually an artifact, i.e., a fixation product?

Second, have you made any calculation of the number of chlorophylls that would fit inside of one of your little discs?

Granick: About 90,000 chlorophylls would be in a disc 5,000 A in diameter and 65 A thick. With regard to the chloroplast membrane: it looks real and, in fact, most of the membranes in tissues are around 50 to 100 A in thickness, which is about the diameter of one to several protein molecules.

Arnold: I am publishing a paper which shows the polarization of the fluorescence in the living plant is about 3%. You can make some sort of argument from this as to the degree of orientation.

Duysens: Dr. Granick made some remarks that, in a unit, the chlorophyll molecule may be oriented parallel. The polarized light would be preferentially absorbed by oriented units and that would give a strong polarization of about 40% at 680 mμ. Since experiments show a much lower degree of polarization, it seems that either the chlorophyll molecules are not oriented exactly parallel in one unit

or that there is energy transfer between units, which is in contradiction to the concept of a unit.

Granick: If chlorophyll molecules are oriented parallel to each other in units or packets and then these units are in turn oriented at random with respect to each other, one might get a small polarization of fluorescence, but not 40%.

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The Natural State of Protochlorophyll

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It is a generally accepted conclusion that chlorophylls when they occur naturally in plants are in a different chemical state than when they are extracted from the plants and dissolved in organic solvents. This conclusion is based on the differences observed in their absorption spectra, in their stability to light, and in their physiological activities in the two environments. When chlorophylls are extracted from the plant by organic solvents, their spectral absorption shifts to shorter wavelengths and their natural physiological activity is destroyed. To account for these differences it has been assumed that the chlorophylls in the native state are combined with carriers to form chemical units possessing characteristic absorption spectra and physiological activities. For convenience of discussion the complete chemical unit has been called a holochrome (1,2), which term is defined as follows: "Holochrome (Gr. *holos* whole + *chroma* color) is proposed as a term to designate a colored substance as it exists in its natural state within an organism, where the colored prosthetic group is combined or associated with a carrier which alters the physical or physiological properties of the prosthetic group" (1).

A long-time aim of research on plant pigments, especially on chloroplast pigments, has been to determine the chemical state in which the pigments exist naturally. Many attempts have been made to isolate a pure form of native chlorophyll. Whether this has ever been accomplished is questionable because it has never been demonstrated that the preparations obtained act physiologically like natural chlorophyll. Indeed, no easy and reliable physiological test for isolated natural chlorophyll is available. This lack has hampered progress toward the preparation of pure chlorophyll holochrome.

* Postdoctoral Fellow of the United States Public Health Service in the Department of Chemistry, Stanford University, at the time when part of this work was done.

Through our work on protochlorophyll, it has become evident that an approach to this aim may be at hand. It has been possible to remove the protochlorophyll holochrome from the leaf and transform it by illumination to the chlorophyll holochrome. Thus the intact natural pigment has been separated from its biological environment in a physiologically active state. If this unit can be isolated and purified while still retaining its physiological activity, it should be feasible to establish its chemical structure. Fortunately, the test for physiological activity is simple: the protochlorophyll holochrome is

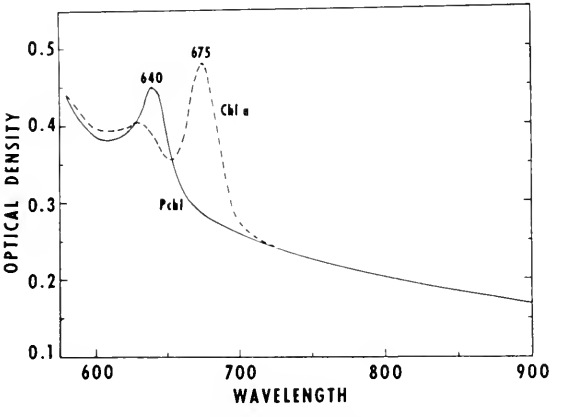

Fig. 1. The transformation of protochlorophyll holochrome to chlorophyll-*a* holochrome in a glycerine extract of etiolated bean leaves.

transformed by light to the chlorophyll holochrome and the conversion estimated by spectrophotometric analysis.

Only a beginning has been made in this project, but the initial results are promising. The purpose of this paper is to describe briefly what has been accomplished in regard to the isolation of the holochrome and to report some of the observations made on the nature of the holochromes.

In our early experiments (3) we obtained extracts of the protochlorophyll holochrome in glycerine. The dark-grown leaves or cotyledons were ground for 10 or 15 minutes in a mortar with from 3 to 4 times their weight of undiluted glycerine. The extract was squeezed through a fine-woven cloth and centrifuged for 10 to 15 minutes at 10,000 $\times$ g. All these operations were carried out in a dark coldroom

at about 6°C. The absorption spectrum of the supernatant was then measured. A portion of the extract, after being illuminated, was again examined spectrophotometrically. Examples of the absorption spectra of the extracts obtained before and after they had been illuminated are shown in Figs. 1 and 2. The chief result of these experiments is that the protochlorophyll holochrome, even though separated from the leaf or cotyledon, is transformed to chlorophyll-*a* holochrome by

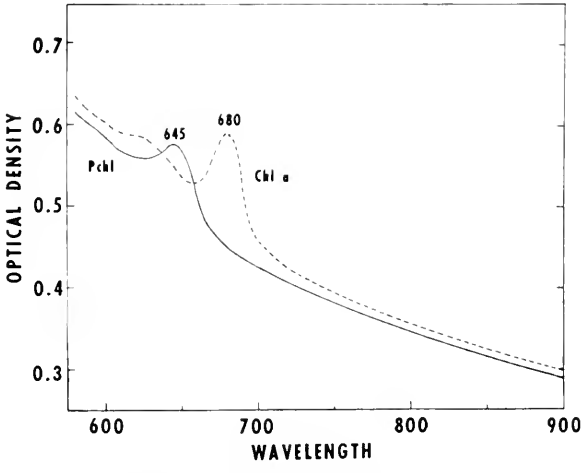

Fig. 2. The transformation of protochlorophyll holochrome to chlorophyll-*a* holochrome in a glycerine extract of etiolated squash cotyledons (Hubbard squash). The glycerine extract was passed through a French-Milner homogenizer (4).

light. These curves also show that the protochlorophyll and chlorophyll holochromes from various sources possess different spectral properties. For instance, in glycerine extracts from different dark-grown plant materials, the absorption maxima of the protochlorophyll holochrome and of the chlorophyll-*a* holochrome derived therefrom lie at different wavelengths: In barley leaves the respective maxima lie at ≈650 and 680 mμ; in bean leaves at 640 and 675 mμ; in squash cotyledons at 645 and 680 mμ. These results indicate that the holochromes from various sources are not identical. They probably differ in respect to the nature of the carrier or of the union between pigment and carrier rather than in the structure of the pigmented component.

Variations occur also in the holochrome from a single plant. Protochlorophyll holochrome extracted by glycerine at about 5°C. from etiolated barley leaves usually has a broad absorption band with an indistinct maximum lying somewhere between 645 and 650 mμ. If this extract is allowed to stand at room temperature in the dark for 1 or 2 hours its absorption maximum gradually shifts to between 630 and 635 mμ (5).

The absorption maximum of the chlorophyll-*a* holochrome shows similar shifts in position. The chlorophyll-*a* holochromes derived from protochlorophyll holochromes with absorption maxima near 650 mμ have absorption maxima that lie near 680 mμ, whereas those derived from protochlorophyll holochromes with absorptions at 630 to 635 mμ usually have absorption maxima near 672 mμ. Furthermore, the chlorophyll-*a* holochrome produced by illumination of dark-grown barley leaves at about 5°C. and extracted in the cold with glycerine had an absorption maximum at 678 mμ. When the extract was kept at room temperature for 3 hours the absorption maximum shifted to 670 mμ. Fully greened barley leaves, however, when extracted with glycerine at room temperature yield an extract whose absorption maximum is close to 680 mμ. The cause of these changes is not known.

A shift in the position of the absorption maximum of chlorophyll *a* from 670 to 677 or 678 mμ during its accumulation in leaves was reported by Krasnovskii and Kosobutskaya (6) who attributed the shift to the change from a monomeric to a colloidally aggregated form of chlorophyll during greening (6,7). Our experiments do not support this interpretation for the shift in the absorption maximum of chlorophyll *a* during greening. When fully greened barley leaves were extracted at room temperature with glycerine we obtained an absorption maximum at 680 mμ. We have also obtained the absorption maximum at 680 mμ for the chlorophyll-*a* holochrome newly formed from protochlorophyll holochrome extracted with cold (≈5°C.) glycerine from etiolated barley leaves and squash cotyledons. These results show that, even without the increase in chlorophyll concentration caused by greening of the leaf, the absorption maximum of chlorophyll *a* lies at 680 mμ—a position comparable to that of chlorophyll in fully greened leaves (8). When the glycerine extracts of newly formed chlorophyll stand at room temperature for some hours, the absorption maxima shift to shorter wavelengths,

e.g., near 672 mμ. With aqueous buffer extracts the shift was very rapid; this may account for the short wavelength position found by the Russian workers. It is very doubtful, therefore, whether a shift in the absorption maximum of native chlorophyll results from chlorophyll accumulation during the greening process.

Although an active form of the protochlorophyll holochrome can be extracted from etiolated leaves by glycerine, such extracts are difficult to work with for the purpose of isolating the holochrome. Because of the technical difficulties involved in the use of glycerine,

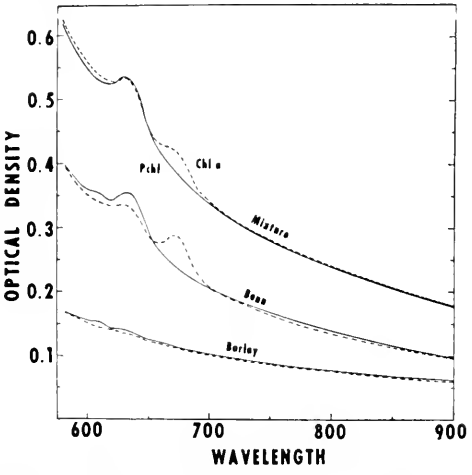

Fig. 3. The transformation of protochlorophyll holochrome to chlorophyll-*a* holochrome in phosphate buffer extracts of barley and bean leaves and of a mixture of the two. The buffer solution, pH 7.08, contained 0.02 *M* phosphate and 0.01 *M* potassium chloride.

we have undertaken the isolation of the holochrome from aqueous buffer extracts of the dark-grown leaves. Krasnovskii and Kosobutskaya (6) were the first to obtain active extracts of the holochrome in an aqueous medium. They extracted etiolated bean leaves with phosphate buffer of pH 7. We have confirmed their results, but we have found that, of the three sources which we examined (barley leaves, bean leaves, and squash cotyledons), only bean leaves yield an active protochlorophyll holochrome in aqueous buffer solutions (Fig. 3). Barley-leaf extracts in phosphate buffer, pH 7.08, contain practically no protochlorophyll and show no transformation. They

appear even to contain an inhibitor for the transformation, inasmuch as an extract of a mixture of barley and bean leaves caused a lessening of the transformation of bean-leaf protochlorophyll holochrome (Fig. 3).

Our experiments on the partial isolation of the active protochlorophyll holochrome will now be described. From 8 to 10 g. of etiolated

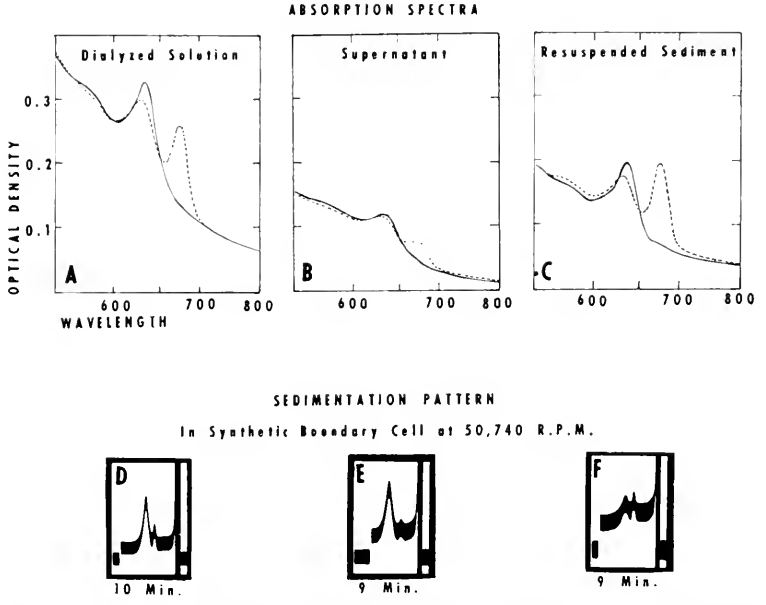

Fig. 4. The photochemical properties and sedimentation patterns of a glycine buffer extract of etiolated bean leaves: A, D before being centrifuged at high speed, B, E the supernatant, and C, F the sediment after 3 hours centrifugation at 40,000 r.p.m. The sediment had been resuspended in fresh buffer. Glycine buffer: 0.1 *M* glycine, 0.05 potassium hydroxide; pH, 9.5. In A, B, and C the full line refers to the unilluminated solutions and the broken line to the illuminated solutions. In the sedimentation patterns, sedimentation proceeds from left to right.

bean leaves were harvested about two weeks after planting and were ground for 15 minutes in 30 ml. of glycine buffer, pH 9.5, containing 0.1 molar glycine and 0.05 molar potassium hydroxide. Glycine buffer was used because the extracts were more stable in this medium than in phosphate buffer. The extract was squeezed through a fine-woven cloth, centrifuged for 10 minutes at 10,000 × g to remove

debris, and the supernatant passed through a French-Milner homogenizer (4). The liquid was then centrifuged for 1 hour at 10,000 × g; exploratory experiments in the analytical ultracentrifuge had demonstrated that this centrifugation removed three relatively fast-moving inactive components. The supernatant was dialyzed against 50 times its volume of glycine buffer. In Fig. 4*A* are shown the absorption spectra of the dialyzed solution before and after being illuminated. Sedimentation analysis of this solution by means of a Spinco Model E ultracentrifuge consistently showed the presence of two sharp boundaries with characteristic sedimentation rates (Fig. 4*D*).

After the dialyzed solution had been centrifuged in the Spinco Model L centrifuge for 0.75 hour at 40,000 r.p.m. (approximately 95,000 × g) most of the holochrome was left in the supernatant solution, but after a 3-hour centrifugation only a small fraction of the pigment was left in the supernatant. This is shown in Figs. 4*B* and 4*E* in which the heights of the absorption peak (Fig. 4*B*) and of the right-hand peak (leading boundary) in the ultracentrifuge pattern (Fig. 4*E*) are both greatly reduced. The small amount of protochlorophyll in this solution is still capable of being transformed by light, as the broken line of Fig. 4*B* shows.

The pellet obtained by the 3-hour, 40,000-r.p.m. centrifugation of the dialyzed solution was washed once with buffer solution and resuspended in a volume of buffer about one half that from which the pellet came. This resuspension required thorough stirring and standing for several hours. The solution so obtained showed the characteristic absorption of the protochlorophyll holochrome before being illuminated and of the chlorophyll-*a* holochrome after being illuminated (Fig. 4*C*). The sedimentation picture of the resuspended material (Fig. 4*F*) obtained with the ultracentrifuge exhibited the two peaks characteristic of the pictures presented in Figs. 4*D* and 4*E*. However, the left-hand peak (trailing boundary) of Fig. 4*F* is much reduced in size as compared to the corresponding peak obtained with the supernatant solution (Fig. 4*E*). The greater portion, therefore, of the particles forming this trailing boundary had not been sedimented during the 3-hour centrifugation. In contrast, the leading peak of Fig. 4*F* is considerably increased over that of Fig. 4*E*, showing that the particles represented by this boundary had been concentrated in the pellet. The correlation between the sizes of the leading boundaries in the sedimentation pictures and of the intensities of the proto-

chlorophyll absorptions makes it clear that the protochlorophyll holochrome was associated with the leading sedimentation boundary and was separated from much of other material in the dialyzed extract by the high-speed centrifugation procedure.

These facts are significant because they demonstrate that the protochlorophyll holochrome can be removed from the plants in an active form which does not require soluble cofactors for its functioning.

The spreading of the boundary associated with the protochlorophyll holochrome during sedimentation was not extensive and appeared to be symmetrical, which indicated no obvious heterogeneity in particle size. The sedimentation coefficient (s_{20}) obtained for several preparations and measured at different stages of preparation ranged from 15.3 to 16.2 Svedbergs. The latter value is probably close to the hypothetical s_{20} at infinite dilution because (*a*) the concentration of active material, although unknown, was very low, and (*b*) artificial sharpening of the boundary could not be demonstrated. Comparison of the s_{20} values with those published for various globular proteins suggests that the molecular weight of the protochlorophyll holochrome is about half a million. This estimate is subject to revision when the other necessary physical constants of the purified particles are obtained. It has been shown that the active particles pass a Millipore Filter which was rated to pass particles less than 0.43 μ in diameter.

One more observation on natural protochlorophyll will be related. During the past year it was found that the protochlorophyll in the leaf is composed of both esterified and unesterified (chlorophyllide) components. Both are transformed by light to the corresponding chlorophyllous derivatives (9). After transformation of the chlorophyllide component to chlorophyllide *a*, esterification to chlorophyll *a* takes place quickly. Thus two paths exist in the normal leaf for the formation of chlorophyll for which the following two schemes are suggested:

1). Protochlorophyllide Holochrome $\xrightarrow{+\text{Phytyl}}$ Protochlorophyll Holochrome $\xrightarrow{+\text{Light}}$ Chlorophyll *a* Holochrome

2). Protochlorophyllide Holochrome $\xrightarrow{+\text{Light}}$ Chlorophyllide *a* Holochrome $\xrightarrow{+\text{Phytyl}}$ Chlorophyll *a* Holochrome

The first of these has already been proposed by Granick (10).

Discussion

Witt: At what temperature were these preparations made?

James Smith: We made them at 6°C. and of course they were done in the dark in order to keep the protochlorophyll from being transformed.

Lumry: As I understand it, you were able to centrifuge your material to the bottom of the tube in a water suspending medium. The density of the material must then be greater than water and thus cannot be high in lipid-containing material.

James Smith: Yes, the density is greater than that of water. One of the properties we still have to determine is the density of the material so that we can get an accurate determination of the molecular weight. If you assume the density to be the same as that of protein, then the molecular weight comes out between 400,000 and 600,000.

Kok: Have you any idea how many magnesium atoms there are in the protochlorophyll holochrome?

James Smith: No, I have no idea about the number of pigment molecules nor of the magnesium atoms in the holochrome. The complete experiment that I have reported here was done just about 10 days before I came and we have had no opportunity to analyze the sedimented particles. We have done many partial experiments and the picture is absolutely consistent in regard to the ultracentrifugation pattern. The time of movement is very constant for this type of biological material, which indicates that we are dealing with pieces of relatively constant properties.

Tolbert: After your first centrifugation at 10,000 × g, the suspension was put through a French-Milner homogenizer. Why did you do that?

James Smith: In order to break down any larger particles so as to get a yield of smaller particles containing protochlorophyll holochrome.

Tolbert: Does this imply that the holochrome protochlorophyll particle is of larger size in the cell than the very small one you isolated? Could you have thrown down a larger particle if you had not put the suspension through the homogenizer?

James Smith: It is possible that the protochlorophyll holochrome exists in the leaf in organized bodies which remain intact during extraction. It was thought that, perhaps by putting the extract through the French-Milner homogenizer, these bodies would be disrupted and their contents dispersed in the solution. Homogenization clarifies the solution to some extent, and this indicates that larger particles are broken down by this treatment. Whether or not the particles that are broken down are the ones that contain the protochlorophyll holochrome is not known because we have never tested the solution by means of the analytical ultracentrifuge before and after homogenization.

Rosenberg: You certainly have shown very beautifully that you are justified in calling this a holochrome because it shows biological properties. Is there any chance of showing some photochemical or physiological property that would justify your calling the transformed material a "holochlorophyll"?

James Smith: Well, we hope that the holochromatic chlorophyll obtained in the manner described will show photochemical physiological properties. You do not have to have physiological activity, however, to call it a holochrome, because

you may still have a linkage between the pigment and the protein, or whatever it is linked to, without having the complex functionally active. The complete pigment may still be present. But our protochlorophyll holochrome certainly is physiologically active in respect to this particular reaction, namely, its phototransformation to chlorophyll holochrome.

Lumry: There appears to be a simple picture in your ultracentrifuge studies in that the preparative technique does not result in a heterogeneous collection of fragments of different sizes but rather a monodisperse material of some constant intrinsic molecular weight.

James Smith: I think that is true and that is Kupke's interpretation. He has said that he would never have expected to have seen the sharp ultracentrifuge pictures and consistent s_{20} values that we have from such preparations if the material were not a fairly homogeneous substance.

Lumry: But remember Emil Smith's detergent-suspended fragments.

James Smith: Detergent-suspended protochlorophyll holochrome will not transform. We have tried many detergents.

Lumry: I know E. Smith's material was beautifully monodisperse with a rather low molecular weight.

James Smith: That may be so. (*Note added in proof*: There is no doubt that with many dispersing agents a great many proteins and structural elements containing proteins will yield smaller monodisperse components even though the starting material is very heterogeneous in size. But this treatment very often causes irreversible denaturation and to my knowledge Emil Smith never claimed that his material possessed physiological activity.)

Arnon: I think this is a very important contribution that we have just heard, because it throws considerable light on the possibility of coordinating this observation with those on the protoplastids which Professors Strugger and Frey-Wyssling have shown to be the precursors of chlorophyll-containing chloroplasts. I take it that you ruled out the possibility that you are merely breaking chloroplasts or larger particles that have not transformed. If this could be coordinated with the physiological picture then you would have a very good idea of just how structural chloroplasts are formed.

James Smith: Of course, to say that you have completely ruled out small organized particles, I suppose, is impossible. But we have filtered the solutions through a Millipore Filter, the finest filter we can get, one which passes particles less than 0.4 μ. This material passes through the filter. This particle is not a big one. I believe it is smaller than a granum.

(*Note added in proof:* Since this paper was presented at the Gatlinburg Conference, we have obtained lyophilized preparations of the active protochlorophyll holochrome. These preparations are slightly yellow and are readily soluble in water. They retain their ability, at least for several months, to form chlorophyll-*a* holochrome when they are illuminated either in the solid state or in solution.)

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The Enzymatic Synthesis of Uroporphyrin Precursors*

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The interesting observations reported by Dr. Smith at this meeting reveal some important facts about final steps in the formation of chlorophyll. The present report will deal with studies on some early steps in the biosynthetic chain of chlorophyll and the other biologically important porphyrins, including porphyrins which are the prosthetic groups of the cytochromes which have received so much attention at these sessions.

Shemin and his co-workers (1,2) have contributed much to the understanding of the roles of glycine and of Krebs cycle intermediates in the synthesis of δ-aminolevulinic acid (DAL) (Fig. 1) and have shown that this compound is utilized in the biosynthesis of protoporphyrin and heme. Workers in several laboratories (3–5) have demonstrated, with enzymes prepared from various sources, the *in vitro* condensation of two molecules of DAL to form one of the monopyrrole porphobilinogen (PBG) (Fig.1). This condensation thus effectively removes DAL from the general metabolic currency of the cell and commits it to use in porphyrin biosynthesis, for it has been shown that the enzymatic conversion of PBG into porphyrins, including protoporphyrin IX, can be catalyzed by cell-free preparations of *Chlorella* (6), hemolyzed avian erythrocytes (7), or other tissues.

The present work has been concerned with the steps involved in the condensation of four molecules of PBG to make the first cyclic tetrapyrrole in the biosynthetic chain under consideration.

The simplest tetrapyrrole which could be formed from four mole-

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cules of PBG is one which could give rise to uroporphyrin I (Fig. 1). The tetrapyrrolic precursor of this porphyrin could be visualized as being formed by the linear condensation of four PBG molecules, followed by a head-to-tail condensation of the linear tetrapyrrole, leading to cyclization. Uroporphyrin I has been found in nature but thus far only in abnormal situations like congenital porphyria in man and its equivalent in other animals; in these cases the compound is excreted by the organism.

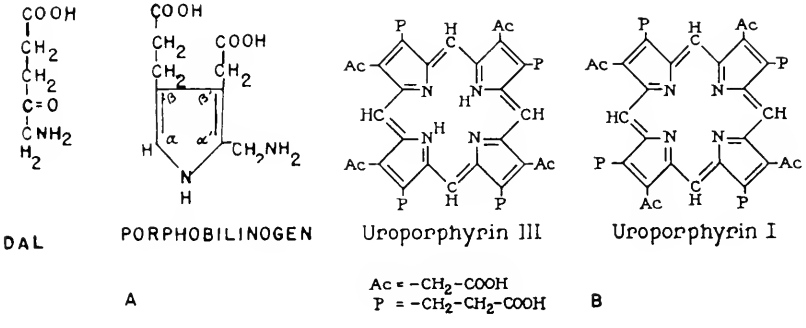

Fig. 1.

In the biosynthetic chain of chlorophyll and protoporphyrin the most probable first tetrapyrrole is one which could give rise to uroporphyrin III (Fig. 1). This compound could not be formed as a product of the linear condensation of four molecules of PBG. Thus this is the "fork in the road" in the utilization of PBG—one branch leads to a product which is useful to the organism as a raw material for the synthesis of porphyrins important in its metabolism; the other leads to a product which, so far as is now known, is of no use to the organism.

Porphobilinogen deaminase has been purified from aqueous extracts of spinach-leaf acetone powder; this enzyme catalyzes the consumption of PBG. (The quantitative estimation of PBG is accomplished by the Ehrlich test in which the concentration of a complex between the pyrrole and *p*-dimethylaminobenzaldehyde is measured colorimetrically. The *p*-dimethylaminobenzaldehyde couples with the pyrrole at the unsubstituted α-position; thus a "consumption of PBG" really means a decrease in the number of α-positions capable of coupling with the *p*-dimethylaminobenzaldehyde.) Concomitantly,

a mole of ammonia is released for each mole of PBG consumed. The reaction proceeds aerobically or anaerobically; and, at the time of exhaustion of the PBG, there is present in the reaction mixture a colorless compound which does not react with *p*-dimethylaminobenzaldehyde, plus, usually, a small amount of porphyrin.

This colorless compound may be spontaneously converted to uroporphyrin I on standing in air (i.e., on oxidation), or it may be converted to this compound by an enzyme of undetermined specificity which is also present in extracts of spinach-leaf acetone powder. The enzymatic conversion proceeds via a short-lived intermediate characterized thus far only by its strong absorption at about 500 mμ.

The uroporphyrin present in the reaction mixtures at the termination of the experiments accounts for 50% to 75% of the PBG consumed; the fate of the remaining PBG has not been determined. The most active PBG deaminase preparations obtained to date have been capable of catalyzing the consumption of approximately 1.3 μM of PBG per milliliter per hour per milligram of protein, at 37°C. The porphyrin produced was characterized as uroporphyrin I by its absorption spectrum, by paper chromatography of the free porphyrin, by paper chromatography of the methyl ester, and by determinations of the melting points of the uroporphyrin methyl ester and of the methyl ester of coproporphyrin prepared by the partial decarboxylation of the uroporphyrin (8). Crystals of the uroporphyrin methyl ester melted at 288° to 289°C., and those of the coproporphyrin methyl ester softened at 235°C. and all birefringence was gone by 252°C.

In contrast to the production of uroporphyrin I from the colorless product of the action of PBG deaminase on PBG, very little of the octacarboxylic porphyrin, uroporphyrin, can be recovered after a solution containing this colorless compound has been incubated with a frozen and thawed preparation of *Chlorella*. Under these circumstances a tetracarboxylic porphyrin, coproporphyrin, is usually found as the major product, and porphyrins with from seven to three carboxyl groups per molecule have been identified on paper chromatograms. These data indicate that this colorless compound may serve as a substrate for the enzymes catalyzing the decarboxylation of precursors of porphyrins like coproporphyrin and protoporphyrin. That the colorless compound is not converted to uroporphyrin prior to decarboxylation is indicated by the observation that

uroporphyrins were not decarboxylated upon incubation with frozen and thawed *Chlorella* preparations of the same kind used in the experiments described above (9,10). These observations are summarized in Fig. 2.

By way of further inquiry into this matter, *Chlorella* preparations have been incubated with uroporphyrinogens prepared by reducing the methene bridges of uroporphyrins with hydrogen, using a palladium catalyst. Small amounts of porphyrins with fewer than eight carboxyl groups per molecule have been recovered, but such experiments have not provided satisfactory quantitative evidence that the bulk of the uroporphyrinogens obtained by this reduction of uroporphyrins are identical with the colorless product of the action of PBG deaminase on PBG.

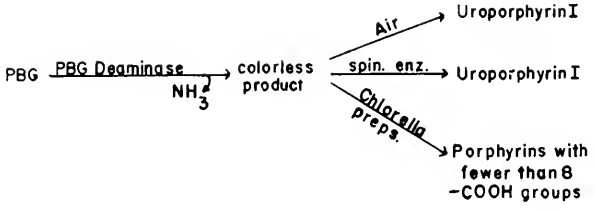

Fig. 2.

The arrangement of the acetic and propionic acid side chains precludes a tetrapyrrolic precursor of uroporphyrin I as a precursor of protoporphyrin IX or chlorophyll. An enzyme has recently been partially purified from wheat germ which plays a role in the biosynthesis of an octacarboxylic tetrapyrrolic precursor of uroporphyrin III; such a compound may be considered the most likely initial tetrapyrrole in this biosynthesis chain.

One fraction prepared from aqueous extracts of wheat germ by ammonium sulfate fractionation catalyzes the consumption of PBG; uroporphyrin III has been recovered from the reaction mixture and characterized by paper chromatography. (Both uroporphyrins III and I have been recovered after PBG has been incubated with some preparations of this fraction.) It is of considerable interest that if such preparations are aged in the refrigerator for 2 days or are heated at 55°C. for 15 minutes, the capacity for the consumption of PBG appears to be unaltered but only uroporphyrin I is produced. (It

should be pointed out that the methods available for the paper chromatography of the methyl esters of the uroporphyrins and coproporphyrins (11) are not capable of distinguishing the I from the II isomer types, or the III from the IV isomer types; however, the melting point data support the contention that the I and the III isomers are the ones being dealt with in these experiments.) These data support some earlier observations (6) that frozen and thawed *Chlorella* preparations which can normally catalyze the synthesis of a number of porphyrins, including protoporphyrin IX, from PBG will catalyze the synthesis of uroporphyrin I almost exclusively if maintained at 55°C. for 30 minutes prior to incubation with the substrate. All these data suggest the participation of two enzymes in the synthesis of the III isomer type as compared with the evidence that the I isomer type is synthesized from PBG in the presence of the deaminase alone.

Another ammonium sulfate fraction of aqueous extracts of wheat germ is of particular interest in this connection. Preparations have been obtained which do not catalyze the "consumption" or other detectable modification of PBG; however, when such wheat germ preparations are incubated with PBG and PBG deaminase and then porphyrin is permitted to form, the product is approximately 85% to 90% uroporphyrin III. This estimate is based on paper chromatography of the uroporphyrin methyl esters, the melting point of the methyl esters (crystals soften at 257°C.; birefringence is gone by 262°C.), and the melting point of the coproporphyrin obtained by the partial decarboxylation of the uroporphyrin mixture (rosettes which soften at 158°C., melt at 199° to 200°C., and recrystallize at 150° to 155°C.). As pointed out repeatedly above, the incubation of PBG with the deaminase alone leads to a product which can be oxidized to uroporphyrin I.

This "isomerizing enzyme" from wheat germ has no effect on the nature of the final porphyrin product if it is added subsequent to the consumption of the PBG in the presence of the deaminase alone, i.e., to a solution containing the colorless product of the reaction by PBG deaminase.

Experiments have also been performed in which PBG was incubated with the wheat germ preparation for 3 hours, and then this enzyme was inactivated by heat treatment (55°C.); this was followed by the addition of PBG deaminase preparations from spinach.

The porphyrin ultimately produced was uroporphyrin isomer I, i.e., there was no indication from the nature of the product that the PBG had ever been exposed to the isomerizing enzyme from wheat germ. The remainder of such an experiment and the nature of the products are shown in Fig. 3; a tracing of a chromatogram of the porphyrin methyl esters is shown in Fig. 4 (12).

Initial contents of reaction mixtures:

#13–#17

0.2 ml. WG 10(40–50); 0.7 ml. 0.5 *M* phos. buffer pH 8.2; 1 ml. PBG (600 mg/ml.); 1.1 ml. H_2O.

#18

As above, but 0.2 ml. H_2O substituted for WG 10 (40–50).

Subsequent additions and manipulations:	*Products:*
13. *a*) Incubated 180 min. at 29°C.	UIII ≫ UI
b) At 180 min., 0.2 ml. 117HIIB added.*	
14. *a*) Incubated 180 min. at 29°C.	
b) Heated 30 min. at 55°C., cooled, 0.2 ml. 117HIIB added.*	UI
15. Incubated at 29°C. for 20 hours.	
16. *a*) Heated 30 min. at 55°C. prior to initial addition of PBG. Cooled, 1 ml. PBG sol. added. Incubated 180 min. at 29°C.	
b) At 180 min., 0.2 ml. 117HIIB added.*	UI
17. *a*) In ice bath 180 min.	
b) At 180 min., 0.2 ml. 117HIIB added.*	UIII ⋙ UI
18. *a*) Incubated 180 min. at 29°C.	
b) Heated 30 min. at 55°C., cooled, 0.2 ml. 117HIIB added.*	UI

* Incubated at 29°C. 17 hours. 117HIIB is a preparation from spinach-leaf acetone powder which contains both PBG deaminase and the enzyme which oxidizes the colorless precursor to uroporphyrin.

Fig. 3.

This requirement for the simultaneous presence of the *deaminase*, of the *isomerizing enzyme* (obtained here from wheat germ), and of *PBG* suggests that perhaps PBG and a polypyrrolic product of the action of the first enzyme (short of a cyclized tetrapyrrole) are both required as substrates for the second (isomerizing) enzyme. A hypothesis regarding the formation of type I and type III isomers proposed earlier by Bogorad and Granick may be pertinent (6).

It appears, from the nature of the porphyrin produced when the

consumption of PBG occurs in the presence of the deaminase alone, that this enzyme catalyzes the "linear" condensation of molecules of the pyrrole with the splitting out of ammonia. It is possible that a linear *tripyrrole* (e.g., Fig. 5) formed through the action of the deaminase on PBG (*E-1*, Fig. 5) constitutes one of the substrates for the isomerizing enzyme which then introduces a molecule of PBG at point "*G*" on the tripyrrole to form a tetrapyrrole of the configuration shown (Fig. 5). A split at *B* of the "*T*" tetrapyrrole would

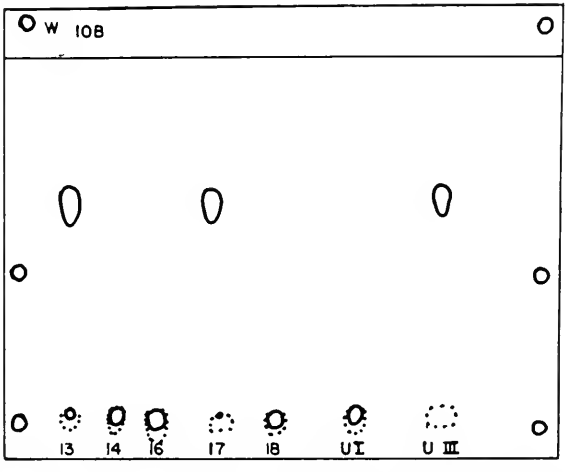

Fig. 4. Tracing of chromatogram of methyl esters of uroporphyrins recovered from expt. W 10B described in Fig. 3. Circles of dotted lines show positions of porphyrins at the end of the first development. Circles of solid lines show positions of the porphyrins at the end of the second development. Numbers correspond to those for treatments in Fig. 3; "UI" and "UIII" are reference compounds, uroporphyrins I and III, respectively.

result in the production of two dipyrrolic compounds which could condense with one another only to form a precursor of a type III porphyrin. This precursor would also serve as a substrate for certain decarboxylating enzymes and thus as a precursor of porphyrins with fewer than eight carboxyl groups per molecule, including protoporphyrin IX and chlorophyll. In the absence of the isomerizing enzyme a fourth molecule of PBG would be added to the one end of the tripyrrole and uroporphyrin I would ultimately be produced. This is one hypothesis which may account for the observations reported here.

These remarks have dwelt on the problem of the "fork in the road." Once the PBG has been set on the "correct" fork and the "proper" tetrapyrrole has been formed, many enzymatic steps must occur

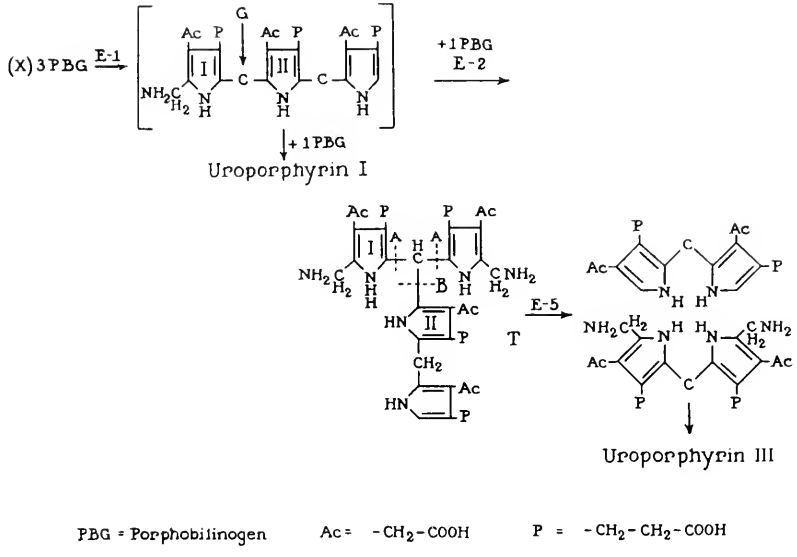

Fig. 5. Hypothetical scheme for the enzymatic synthesis of uroporphyrins (6). According to this hypothesis "E-1" would correspond to PBG deaminase, "E-2" to the isomerizing enzyme.

before the goal—photosynthetically active chlorophyll and biologically active cytochromes—is reached. Detailed maps of a suggested route to this goal, which should perhaps be marked "carry extra water, food, and gasoline," are available elsewhere (e.g., 6,13).

Addendum

The intermediate in the appearance of uroporphyrin from the colorless compound produced by the action of porphobilinogen deaminase on PBG and characterized by its strong absorption at about 500 mμ has been identified as a stage in the oxidation of uroporphyrinogen to porphyrin. The strong 500 mμ band, sometimes accompanied by weak absorption bands at 538, 561, and 612 mμ, appears during the oxida-

tion of uroporphyrinogen to uroporphyrin with iodine under the proper conditions.

It has been possible also to demonstrate, using slightly modified conditions, the extensive conversion of uroporphyrinogen I, as well as the colorless product of the action of porphobilinogen deaminase on PBG, to porphyrins with fewer than eight carboxyl groups per molecule by frozen and thawed preparations of *Chlorella*. The quantitative extent of the conversion is the same in the case of both substrates. These data, plus those given in this paper, support the conclusion that the colorless product of the action of the deaminase on PBG is uroporphyrinogen and most of it is of the I isomer type.

When the deaminase, a preparation of the isomerizing enzyme, and PBG are incubated together under anaerobic conditions and in the presence of cysteine, little or no porphyrin appears although the substrate is consumed. The incubation of this material, in which the PBG is exhausted, with the frozen and thawed *Chlorella* leads to the formation of porphyrins with fewer than eight carboxyl groups per molecule, including protoporphyrin. These *Chlorella* preparations are capable of catalyzing the production of the same porphyrins when uroporphyrinogen III is supplied as the substrate. Thus the unoxidized product of the PBG-deaminase-isomerizing enzyme system appears to be uroporphyrinogen III. (Also see: Neve, R. A., Labbe, R. F., and Aldrich, R. A., *J. Am. Chem. Soc.*, *78*, 691 (1956).)

Discussion

Vernon: Have you made any models of this tetrapyrrylmethane?

Bogorad: Well, let me say that tripyrrylmethanes of this same general configuration have been synthesized. Now, the only difference between this postulated tetrapyrrylmethane and the tripyrrylmethanes which have been synthesized, besides the added pyrrole, is in the side chains which would probably not interfere stearically. Tetrapyrrylmethanes of the kind we have postulated have never been seen except on paper.

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On Uniformity of Experimental Material

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In the use of algae such as *Chlorella* and *Scenedesmus* as experimental material, uniformity has two aspects: reproducibility and homogeneity. Reproducible photosynthetic behavior, as observed within a short time span, is readily obtained from replicate aliquots of a harvested suspension. However, harvests taken at different times from a batch culture are known to show wide variability in photosynthetic characteristics. With increasing density of a culture the average light intensity per cell continually decreases. Light intensity and all other conditions which are functions of cell concentration may be controlled and stabilized by use of steady-state culture devices which maintain cell concentration constant by controlled or continuous dilution. Our continuous-culture apparatus employs a photometric system to dilute a culture as fast as it grows and hold constant the cell concentration (1). Both in theory and in practice it yields reproducible experimental material day after day. Its operation is stable except under limitation of growth by a nutrient deficiency in the diluting medium. An inverted kind of steady-state device is the chemostat of Novick and Szilard (2) which employs a constant dilution rate; here operation is stable only if some medium component limits the growth rate. It might be useful for producing algae with a constant nutrient deficiency although it would be sluggish in reaching steady-state operation because of the rather low growth rates of most algae.

Use of steady-state devices such as the continuous-culture apparatus yields harvests in which there is a constant frequency distribution of cells at the different stages of their life cycle. The cells are not homogeneous with respect to "age" and may not be homogeneous in photosynthetic characteristics. That this is indeed the case has been shown by Pirson and co-workers for *Hydrodictyon* (this symposium), by Tamiya and co-workers for *Chlorella ellipsoidea* (3)

and by Sorokin in our laboratory for a high-temperature strain of *Chlorella*.

In the life cycle of *Chlorella* a small cell increases in size by assimilation to a large cell which divides into n small cells (autospores). In *C. ellipsoidea*, *C. pyrenoidosa*, and the high-temperature *Chlorella* Tx71105, the number n lies somewhere between 4 and 8, although it has a statistical rather than a fixed value.

To depart from the main theme of this discussion, there should be noted the exceptional case of the Emerson strain of *C. vulgaris* which grows very poorly in the dark on glucose medium. Since its cells then become large and packed with starch there appears to be no difficulty in glucose assimilation in the dark. A light dosage so small as to give no significant growth in the absence of glucose will greatly increase the rate of growth in the presence of glucose and will prevent the formation of unusually large cells. We interpret the phenomenon as a light requirement or stimulation of the division phase in *C. vulgaris*. However, the phenomenon is not shown by other *Chlorella* and *Scenedesmus* strains examined.

For cells growing photosynthetically our experience confirms the conclusion of Tamiya: only the assimilation phase is light-dependent. By a judicious regimen of light and dark periods it is possible to obtain suspensions of cells which are nearly homogeneous or synchronized with respect to division cycle.

In our laboratory Sorokin has resolved the time course of changes in photosynthetic activity occurring when a synchronized suspension of small cells of his Tx71105 are illuminated at 30°C. and light saturation over an 8-hour period. Light-saturated rate of photosynthesis per dry weight of cells rises rapidly to a maximum at about 3 hours and then falls continuously. After about 8 hours, the cell number begins to rise, signifying the beginning of divisions. There are accompanying changes in chlorophyll concentration, respiratory rates, and photosynthetic quotient. (*Note added in proof:* Following the Gatlinberg conference there became available a report from Tamiya's laboratory (4) detailing changes in the life cycle of *C. ellipsoidea*.)

Variation accompanying the life cycle is not peculiar to the algae but occurs also in other microorganisms. The problem may be likened to a wave phenomenon, in this case a plot of volume of a cell vs. time. In a bacterium such as *E. coli*, dividing rapidly by binary fission, the waveform has an amplitude of 100% of the minimum and

a frequency of about 50 per day. In *C. ellipsoidea* and *C. pyrenoidosa* the waveform has an amplitude of up to 700% of the minimum and a frequency of 1 per day or less. The large amplitude and low frequency in the *Chlorellas* present special problems to the attainment of uniform experimental material. The amplitude of physiological variation appears less than that of cell volume but heterogeneous material will always result unless cultures are carefully synchronized for division cycle. In conventional batch cultures nonreproducible material may result from unintentional synchronizing and variation in stage of division cycle at harvest unless there is a very strict regimen.

Two methods of obtaining both homogeneous and reproducible material appear feasible. Tamiya's laboratory has used a technique reminiscent of the training of cells for quantum yield measurements: cultures are developed at high light intensity and then incubated for 7 days at low intensity; resultant cultures have over 90% of small cells of high photosynthetic activity. In our laboratory Sorokin has imposed a careful regimen of light and dark periods upon the otherwise steady-state operation of the continuous-culture apparatus. It appears that the increasing elegance of measurements applied to photosynthesis merits similar attention to uniformity of experimental material.

Discussion

Aronoff: I am very glad that we have a system now which parallels in many respects the advantages of a young leaf and still possesses the statistical advantages of a large population. In the leaf one has at the moment of its emergence all the cells which the leaf will subsequently have. There is no further mitotic activity. We have conducted age studies on such leaves and have shown that the rate of synthesis of essential amino acids is very different in young leaves as compared to old leaves. It is, therefore, not unreasonable that the composition of such things as proteins is continuously changing throughout the life cycle of leaves, and such a phenomenon, while not unexpected, is in accord with the data that have been found for these algae.

Tolbert: In older algal cultures, say 3 days old, in a growth flask, if the growth rate is slowed down are there possibly larger cells?

Myers: In a dense culture two kinds of limitations may develop. A light intensity limitation will give rise to a greater proportion of small cells. A nutrient limitation generally has the opposite effect and gives rise to increase in proportion of large cells.

Tolbert: In much of previous photosynthesis research with *Chlorella*, algal cultures ranging from 1 to 3 or 4 days old have been used after having been grown

under various conditions of light intensity, temperature, and CO_2 partial pressure. What can be predicted as to the type or size of cells that are used?

Myers: I don't know any *a priori* reason to predict what this situation will be.

Tolbert: Is there any relationship between photoperiodism in higher plants and the dependence on light for cleavage of the larger *Chlorella* cells to smaller cells?

Myers: That, of course, is the reason this point becomes interesting. All I can say is that stimulation of cell division is accomplished by an amount of light which is below the compensation point as determined by growth. It is too small to make any significant contribution to carbon assimilation via photosynthesis. Furthermore, cultures of *Chlorella vulgaris* grown in the dark have remarkably large cells and show unusual division phenomena.

Tamiya: As Dr. Myers stated, we distinguished at the beginning of our life-cycle studies only two types of cells: the smaller ones which we called dark cells and the larger ones which we called light cells. But, since we investigated the change of physiological activities occurring during the life cycle of algae, we found the necessity for more detailed discrimination of developmental stages. Now we distinguish two stages in dark cells ("nascent" and "active") and three successive stages in light cells (stages 1, 2, and 3). The light cells at different stages are almost the same in their size, but markedly different in their grade of ripening or their capacity for forming autospores when kept dark under aerobic conditions. Chlorophyll content and photosynthetic activity are highest in "active" dark cells and lowest in the light cells at stage 2. On the other hand, the respiratory activity is lowest in "active" dark cells and highest in the light cells at stages 2 and 3. I think that the data presented by Dr. Myers may well be understood on the basis of our observations.

Pirson: I want to make a short remark on some of our experiments with *Chlorella*. We put *Chlorella* (this was the Emerson strain, not the strain used by Professor Tamiya) in light and dark—12 hours dark and 12 hours light. In one light period we had no cell division, only an enlargement of the cells, and in the other light period it was found that a very extensive cell division occurred and the cell size decreased.

As long as the suspensions were very thin and the intensities very high, we got a characteristic periodic response of photosynthesis independent of cell division. It consisted of a regular decrease during the time of illumination. We believe, therefore, that we have another periodic principle which may be related to that observed by Professor Tamiya. But this holds true only in very thin suspensions, where photosynthesis cannot be measured manometrically. We have to make these determinations using the polarographic method.

Wassink: I understand that your curve at the maximum of 130 volumes of oxygen per cell volume per hour refers to experiments on light saturation.

Myers: That is correct.

Wassink: Have you determined whether anything happens to the quantum efficiency at that point?

Myers: We have not determined quantum efficiencies. However, when a synchronized culture of small cells is illuminated there follows a characteristic time course in the shape of the light intensity curves of photosynthesis observed in

cells removed from the culture. I regret that I am not prepared to draw such curves for you.

French: If you centrifuge out little cells from big ones from a continuous culture do you get much difference in the growth or photosynthetic rates and chlorophyll content?

Myers: We have not tried to do that. Professor Tamiya has been doing it. Perhaps he can answer your question better than I.

Tamiya: By centrifugal fractionation they can be separated to some extent, but not very sharply.

French: But are the differences in the cells as great as though they were produced by modifying the culture conditions?

Tamiya: Maybe so, if we repeat the fractionation many times; but to achieve this we must expect to lose a very large quantity of algal cells.

Emerson: I have an answer for Professor Wassink. We have measured quantum efficiency of cells in different stages of development. Lewis and I did this by fractional centrifugation, and recently we have been doing it by controlling the periodicity of light and darkness during culture growth. From the evidence obtained by fractionation of a given batch of cells, the steady-state quantum efficiency seems to be about the same for large and small cells, but the transients at the beginning of the light and dark periods may differ greatly at successive stages of development of the cells, and if the transients are included in calculation of quantum yields, then large differences in yield are easily obtainable.

Wassink: How long do these transient periods of rates of metabolism last? Just seconds or minutes?

Emerson: According to our evidence from manometric experiments, these transients last for several minutes.

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Induced Periodicity of Photosynthesis and Respiration in *Hydrodictyon*

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The comparison of highly organized algal cells with cells of the simple unicellular green algae as standard objects of photosynthetic research led us to investigations of photosynthesis and aerobic respiration of *Hydrodictyon*, which, by previous examinations in our laboratory (1), proved to be suitable for simultaneous measurements of morphological, cell-physiological, and metabolic properties.

Hydrodictyon forms netlike colonies; the meshes of the loose network are formed by single cells. Growth of these colonies does not occur by cell division, but entirely by an easily determinable elongation of the cells, in the course of which the cells change over from the young mononuclear stage into a multinuclear one. The chloroplast forms are more or less perforated hollow cylinders. After the cells have reached a certain length, the numerous nuclei of each cell can be caused by simple methods, e.g., temporary treatment with strong light, to form zoospores together with a small portion of cytoplasm and plastid material. Normally these zoospores are not freed but unite after a short time within the mother cell, forming a daughter net, which, after a rapid disintegration of the mother membrane, is liberated and then starts the vegetative cycle again. The numerous cells of a net yield quite a homogeneous material with but little physiological deviation. Besides the vegetative cycle, *Hydrodictyon* also exhibits a sexual cycle, which, however, can be completely eliminated by suitable culturing conditions. In any case, growing this alga requires more supervision than does culturing algae of the *Chlorella* type. As the concentration of the nutrient medium (solution of Uspenski) must be kept low, a frequent change of solution is necessary in order to avoid mineral deficiencies.

Among the physiological complications brought about by the higher morphological differentiation it is particularly remarkable that *Hydrodictyon*, in contrast to *Chlorella*, requires a regular alternation of light (400 to 800 lux) and darkness for its normal develop-

ment. When growing the alga in continuous light, characteristic growth anomalies can be observed after a few days. These lead to hourglass-shaped cells and later to irreversible damages. An alternation of light and darkness (e.g., 12:12, 24:24, 6:6 hours) completely prevents such anomalies.

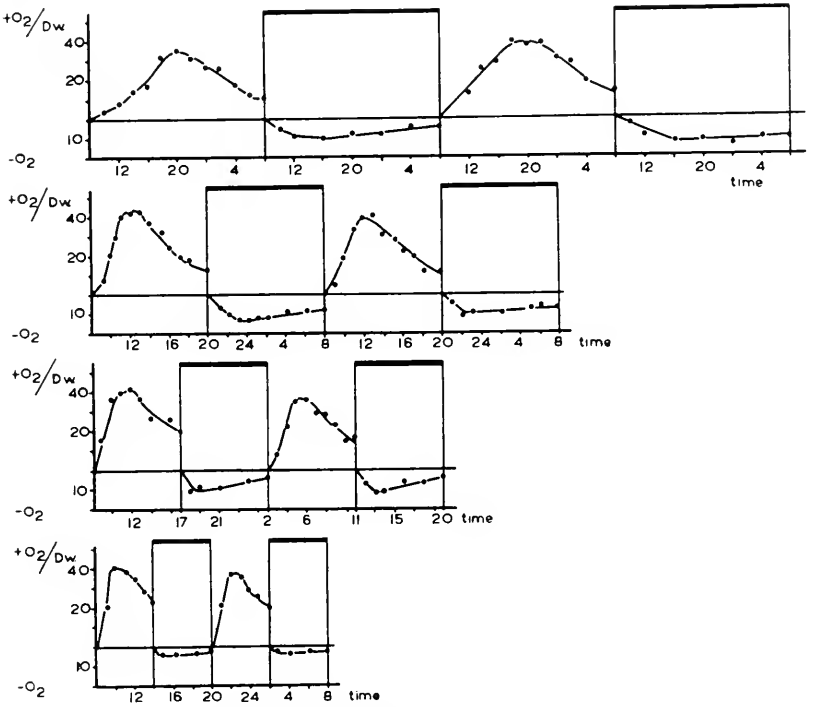

Fig. 1. Time course of photosynthesis and respiration of *Hydrodictyon* at different lengths of light and dark periods. Ordinate: O_2 production and respiratory O_2 consumption expressed in mm. galvanometer deflection per time and dry weight.

As *Hydrodictyon* is not suitable for manometric measurements, photosynthesis and respiration were determined by the polarographic method (dropping mercury electrode). Photosynthetic O_2 production during the normally applied 12-hour light period is not at all constant. Maximum production is reached about 4 hours after the start of illumination, whereupon follows a marked decrease to about one-third of the maximal rate. After switching off the light, the respiratory

O_2 consumption at first is not maximal, but rises during the first 4 hours of darkness and then gradually decreases toward the end of the dark period. Thus we observe, in connection with light-dark changes, a periodically changing activity of photosynthesis and respiration. This periodicity is not confined to periods of 24 hours, but can vary in a broad range with changes in the length of the light and dark period, as detailed investigations of W. J. Schön (2) in our institute have shown (Fig. 1). The maximum of photosynthesis, in these experiments, is generally shifted into the first half of the light period, the maximum of respiration into the first half of the dark time. An alternation of 3 hours light and 3 hours darkness acts like continuous illumination. In this case, photosynthesis steadily decreases; at the compensation point, the first symptoms of the above-mentioned growth anomalies appear. To a certain extent the length of light and dark periods can also be varied independently of each other; thus even a periodicity of $10^1/_2$:7 hours light and darkness can be imposed upon the alga. Complete adaptation to a new frequency generally needs a pretreatment over about 6 to 8 periods.

These experiments show that metabolic periodicity is not directly connected to the natural change of light and darkness, or to the length of the day (daily sun cycle). It is easily possible to induce an inversion of periodicity by doubling the dark time once. As to a right judgment of the induction of photosynthetic periodicity it should be stressed that the measurements were not continuously performed on one specimen of a *Hydrodictyon* colony but that, during one period, the experimental material was supplied several times from a uniformly treated stock. Determinations of the dry weight of the cells growing during the experiment enabled us to eliminate the increase of photosynthesis caused by newly formed metabolically active substance. In this connection it is important to note that, within the whole light period, chlorophyll content (0.8%) and gross composition of the cells remained fairly constant. This means that the decrease of photosynthetic output occurring in the second half of the light period cannot be based on an accumulation of storage material. We rather have to conclude that a genuine regulation of photosynthetic activity by the cell takes place.

For the analysis of the mechanism of this periodical regulation of photosynthesis not only the above-mentioned shifting of the maximum is important, but also the course of photosynthesis after dark

intervals longer or shorter than the light times. After a pretreatment of 48 hours darkness the rise of photosynthesis during 12 hours light is so much prolonged that the maximal value is not reached until the end of the light time; in a following 12:12 light-dark change, however, the normal periodicity will rapidly return. If the dark time has been chosen shorter than the light time, e.g., 3, 6, or 9 hours with a light period of 12 hours, photosynthesis does not reach its full efficiency; the maximal value then depends almost proportionally upon the duration of darkness. When, during the dark time, the temperature is raised by 10°C. over that of the light time (30°C. compared with 20°C.), the increase of photosynthesis is retarded and the effect is similar to that of very long darkness. If, however, dark respiration is not raised by temperature but by addition of glucose, the normal periodicity of photosynthesis is maintained. In this case, the presence of glucose does not affect the rate of apparent photosynthesis; hence it must be concluded that the marked increase of dark respiration caused by glucose does not take place in light and that the consumption of glucose in light must occur in a way different from that of oxidative dark assimilation. This concept is supported by findings in *Chlorella,* which have been obtained in another way (3,4). On altering the carbon dioxide concentration no change of the photosynthetic periodicity could be induced. Higher light intensities (2000 lux, instead of the normally applied 1000 lux) accelerate the increase of photosynthesis to the maximum; the following decrease is steeper and leads to a smaller final value. At low intensities (150 lux) periodicity disappears; photosynthesis then keeps nearly constant at a low absolute value throughout the whole light period. Hence it may be concluded in the conventional way that periodicity does not affect the photochemical reaction but affects the enzymatic component of photosynthesis.

The hitherto-mentioned data may formally be explained by the relatively simple working hypothesis that respiration and photosynthesis, respectively, develop an inhibitory principle reducing the rate of photosynthesis; it must further be assumed that both principles may inactivate each other. According to this conception, photosynthesis at the beginning of illumination would be limited by the dark principle; the inhibitory factor formed in light would in this phase be canceled out by reacting with the dark component. At the maximum of photosynthesis the dark principle would be com-

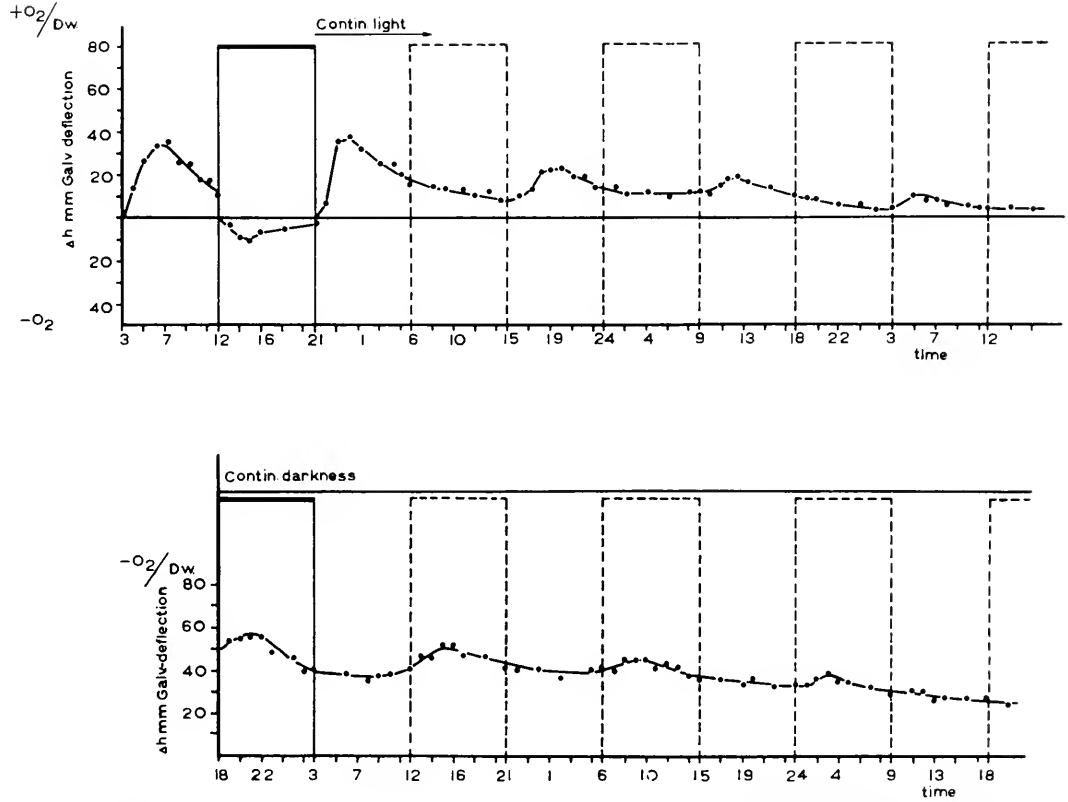

Fig. 2. After-oscillations of photosynthesis (above) and respiration (below) of *Hydrodictyon* with a metabolic periodicity adapted to 18 hours (9 hours light and 9 hours darkness). Dotted lines: periods corresponding to the previously applied dark times.

pletely removed; the light-induced principle, on the other hand, would not yet have accumulated because of the previous continuous destruction; however, accumulation would then occur in the following period of illumination so that photosynthesis would then decrease. Possibly light and dark inhibitors are components of the same redox system. At the beginning of illumination the photosynthetic apparatus would be in an overoxidized state and at the end of the light period in an overreduced one. The maximal value would correspond to an optimal relation of the two redox partners. Dark respiration probably is regulated by an inverse mechanism. The increase of respiration during the first hours of darkness may be caused by a gradual inactivation of an inhibitory principle (formed in light) by means of the respiratory apparatus.

In changing from a light-dark cycle to constant conditions, periodicity is maintained for over 2 to 3 periods. In continuous illumination photosynthesis goes on reacting periodically, as does respiration in continuous darkness. For the explanation of these after-oscillations a simple biochemical mechanism cannot be taken into account. Here we are facing a phenomenon somehow resembling the after-oscillations, which, since W. Pfeffer's observations on the foliar movements of beans, have been described as rhythmically determined reactions in very different organisms. In regard to their frequency, the after-oscillations described by previous authors were independent of the external periodicity applied during pretreatment and thus indicated the presence of an endogenous rhythm, whereas the after-oscillations of photosynthesis and respiration in *Hydrodictyon* behave quite differently. They follow almost exactly the inductive frequency and thus are as plastic as periodicity under the conditions of light-dark change (*cf.* Fig. 1). Further investigations will be necessary to demonstrate the importance of this difference and to give evidence as to whether the observed metabolic periodicities have a direct value for the analysis of the so-called endogenous rhythms of the Pfeffer-type (5). From all our experimental results, only the after-oscillations of photosynthesis and respiration induced by a periodical induction of 9:9 hours have been selected and are shown in Fig. 2. It is particularly interesting that the after-oscillations of photosynthesis in continuous light and those of respiration in continuous darkness do not coincide, but alternate with each other. A maximum of photosynthesis generally corresponds to a minimum of respiration.

Projecting these findings back into the previous conditions of light-dark cycling we must conclude that, in *Hydrodictyon*, respiration is reduced during illumination; this behavior seems to differ from that of *Chlorella* (6). The requirement of intermittent darkening in *Hydrodictyon* perhaps is based upon the fact that dark respiration, which seems to be inhibited in light, possesses an indispensable function in normal development.

Although we cannot find a simple biochemical mechanism explaining the periodical changes of metabolism in constant conditions, we are able to influence the after-oscillations experimentally and thereby characterize the mechanism of periodicity. Thus it is possible to raise respiration to a higher level by addition of 0.01% glucose and thereby eliminate the after-oscillations. Photosynthetic periodicity, however, cannot be altered by glucose, either during intermittent illumination or in subsequent continuous light. By interrupting CO_2 supply during intermittent illumination we observed only very low rates of photosynthesis produced by the stored intracellular respiratory carbon dioxide of the dark times. If this treatment is followed by continuous illumination with sufficient CO_2 supply, after-oscillations are very small. This strengthens the assumption that photosynthetic periodicity is not induced by the change of light and darkness per se, but by a periodically fluctuating activity of the photosynthetic mechanism. It is possible, by the way, to let photosynthesis work quite aperiodically for some time (2 to 3 days). This can be done by treating the alga with constant light for about 48 hours without CO_2 and then supplying it with sufficient CO_2, still keeping illumination constant. Hence periodical changes of photosynthetic activity need an external induction and do not represent a behavior of photosynthesis fixed *a priori* in this alga.

Beside a *physiological* analysis of the periodical metabolic reactions of *Hydrodictyon*, a treatment of periodicity was also started on an *enzymological* basis. It could be shown that the activity of acid phosphatase obtained from extracts of the algal colonies at different times of the light and dark periods has strong fluctuations similar to the periodicity of photosynthesis and respiration (7). The maximum of photosynthesis in light and the maximum of respiration in darkness, respectively, coincide with the minima of phosphatase activity, i.e., the frequency of enzyme periodicity appears to be twice that of the two single metabolic processes (Fig. 3). It would be premature

to deduce definite biochemical relations from this coincidence of metabolic and phosphatase activity. We do not know yet whether the periodical behavior of phosphatase can be regarded as specific, or whether more or less the whole enzymic system of the cell is compulsorily submitted to such fluctuations of activity. This question is being examined at present in our laboratory (*cf.* 7).

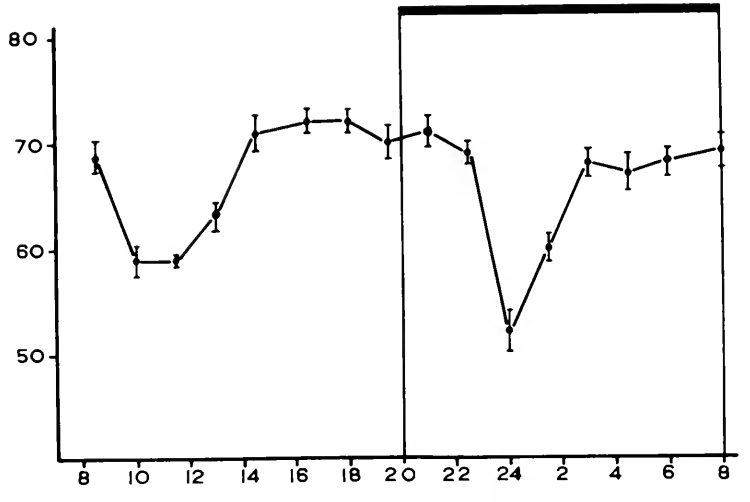

Fig. 3. Periodicity of acid phosphatase (substrate: phenylphosphate) related to protein content in crude extracts of *Hydrodictyon* prepared at different times of light and dark periods. Ordinate: γ phenol/100 γ prot.; abscissa: time.

Periodicity in *Hydrodictyon* is not confined to simple metabolic processes. As a result of metabolic periodicity more complex activities of the cell also exhibit rhythmic variations. Among these, periodic growth rates are most conspicuous. In the light periods, periodicity of growth parallels photosynthetic periodicity almost completely. Accelerations of photosynthesis in continuous light (after-oscillations) correspond exactly to accelerations of growth rates. In darkness the growth rate gradually approaches zero; in continuous darkness immediately following a period of light-dark change we observed two or three small gushes of growth at a time when the light periods had occurred earlier. These growth increases, therefore, seem to be induced by the periodic activity of

photosynthesis and not by the periodicity of dark respiration. These last-mentioned results and similar ones have already been published elsewhere (8).

Discussion

Tamiya: How do you measure growth?

Pirson: Only by measuring elongation, but we know that at the time of elongation plasmatic growth takes place also.

Witt: Can you also change the period by periodically varying carbon dioxide?

Pirson: This may be possible and it is on our program (*cf.* 2a).

Witt: Can you change the period, by periodically changing the temperature between 30°C. and 5°C.?

Pirson: It would be fine if we could do that, but if we go to temperatures of about 5°C. we observe a long time before recovery. Then we cannot decide whether the effects are recovery effects or periodical effects.

Wassink: I would like to make an additional comment on this etiolation question. It seems dangerous to use the term etiolation, unless specifically attached to the absence of light. You may know that a considerable time ago we published evidence that, for instance, in lettuce seedlings you can easily make a light regimen, say 10 hours white light and 14 of darkness, that will produce no etiolation or at least no appreciable elongation in leaves and stems. On the other hand, if you supply this basic light period with, say, 8 hours of weak intensity of either near infrared or the blue-violet region, you will find that the plant will show etiolation characteristics. So it seems that we have to deal here with special photomorphogenetic phenomena and not only with the absence of light. You might be able to produce conditions similar to ours, in which you really would find etiolation in terms of elongation by supplying your cells with special regions of light; and thus we will now have to say more precisely what we mean by etiolation.

Pirson: Your criticism of the use of the term etiolation is quite right. I said they grow very little in the dark.

Rabinowitch: I just want to ask whether your medium was constant; was it a flowing medium, or was there any periodic change in the medium involved?

Pirson: It was not a flowing medium, but the solution was renewed several times during one period. Hence we can exclude the possibility of a change in the medium. It should be stressed that the cells suffered no deficiencies during our experiments. Photosynthesis and respiration were related to dry-weight; for this purpose the samples were changed every 3 hours. The chlorophyll content remained constant on a dry-weight basis. This means that there was no change between periods of activity and storage periods.

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