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Photooxidation of Cytochromes c, f, and Plastocyanin by Detergent Treated Chloroplasts [1, 2]

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Introduction

Nieman et al. (16, 17) and Bishop et al. (2) described the photooxidation of ferrocytochrome c by detergent treated chloroplasts as an oxygen-consuming, cyanide-insensitive process which was stimulated by a soluble chloroplast enzyme. In an earlier paper, we reported that the quantum yield of this reaction increases at wavelengths beyond 690 mμ (14), and concluded that only the first photoact is operative which is sensitized by chlorophyll a and in which P700 (10) serves as the primary energy converter.

$$X + P700 \xrightarrow[\leftarrow\!-\!-k_b\!-\!-]{h\nu,\ \text{chlorophyll a}} X^- + P700^+ \quad \text{I, Ib}$$

$$Fe^{++}\text{cyt} + P700^+ \xrightarrow{k_2} Fe^{+++}\text{cyt} + P700 \quad \text{II}$$

$$X^- + \tfrac{1}{4}\,O_2 \xrightarrow{k_3} X + \tfrac{1}{2}\,H_2O \quad \text{III}$$

Reaction sequence I to III describes this hypothesis: the light-driven electron transfer from P700 to X yields a weak oxidant, $P700^+$, and a strong reductant, X^-. [Normal potential +0.43 v (10) and <-0.45 v, respectively.] The primary photooxidant oxidizes cytochrome c and the primary photoreductant is reoxidized by O_2. The primary photoproducts recombine in a back reaction (k_b) if the two finishing dark reactions cannot keep pace with their formation.

This paper describes further observations concerning this reaction sequence with particular emphasis on cofactors enhancing the rate of cytochrome c oxidation and their mode of action.

Materials and Methods

All experiments reported were made with detergent treated spinach chloroplasts prepared as follows: to 1 ml freshly prepared and washed chloroplasts, suspended in Tris buffer pH 7.8 in a concentration of 1 mg chlorophyll per milliliter, 5 % Tween 20 (Atlas Co.) was added. After ~2 minutes standing, the sample was diluted to 10 ml and sonicated during 5 minutes in a Raytheon oscillator. Activity did not vary much with variation of procedural details, including removal of the detergent after sonic treatment by dialysis.

Rates of substrate oxidation were measured in a double beam spectrophotometer which allowed the illumination of the sample while recording its optical density: by means of a synchronously revolving mirror the monochromatic detecting light was passed alternately through the sample and a reference cuvet. During the transition of this beam from one cuvet to the other (60 times per sec) the photomultiplier tube was darkened and a strong actinic beam admitted during 6.8 milliseconds to the sample cuvet.

Intensity of the actinic light was measured with an integrating device described earlier (14) and fractional absorption of the suspension with a double beam integrating sphere.

Results

The Role of P700 as the Photoconverter. In a previous paper, a method was described for the partial purification of P700 using dilute acetone solutions to extract most of the chlorophyll from algae or chloroplasts (10). The material thus treated photooxidizes ferrocytochrome c at moderate rates.

Four aliquots of detergent treated chloroplasts were briefly subjected to various concentrations of acetone-water mixtures, centrifuged, washed, and resuspended in their original volume of buffer. The amount of chloropyll remaining was determined by exhaustive extraction with 80 % acetone and determination of the optical density of the extract at 665 mμ. The relative amount of P700 was measured as the magnitude of the light-induced reversible absorption change at 700 mμ in the presence of 10^{-5} M PMS[3] (12). Finally, the rate of cytochrome c oxidation in light of saturating intensity was determined in the presence of 10^{-4} M benzyl viologen.

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[3] Abbreviations: PMS, phenazine methosulfate; FMN, flavin mononucleotide; FAD, flavin-adenine dinucleotide; DCMU, 3-(3,4-dicholorophenyl)-1,1-dimethyl urea; PC, plastocyanin; DCPIP, 2,6-dichlorophenol-indophenol.

Figure 1 shows the data plotted as percentages of the control values. The photooxidation activity parallels closely the amount of P700 remaining in the sample. Both quantities decline suddenly if the acetone concentration is raised from 70 to 75%. Thus, participation of the pigment in cytochrome oxidation is demonstrated.

Stimulation by Viologen Dyes; Inhibition by PMS. Early in this investigation it was found that both methyl and benzyl viologen stimulate the slow endogenous rate of cytochrome c photooxidation. FMN, FAD, and menadione proved to act similarly. This stimulation is between two- and sixfold and holds true independent of the presence of other catalysts and for low and high light intensities, i.e., not only the saturation rate but also the quantum yield

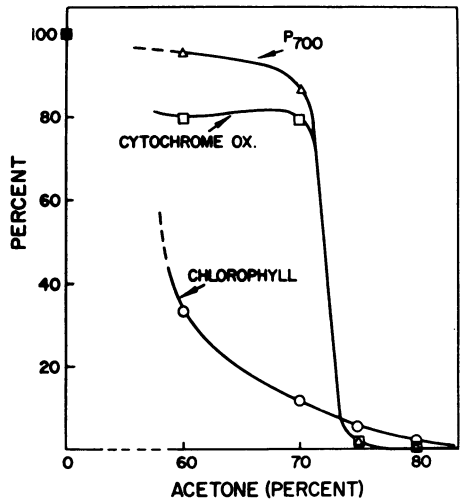

FIGURE 1

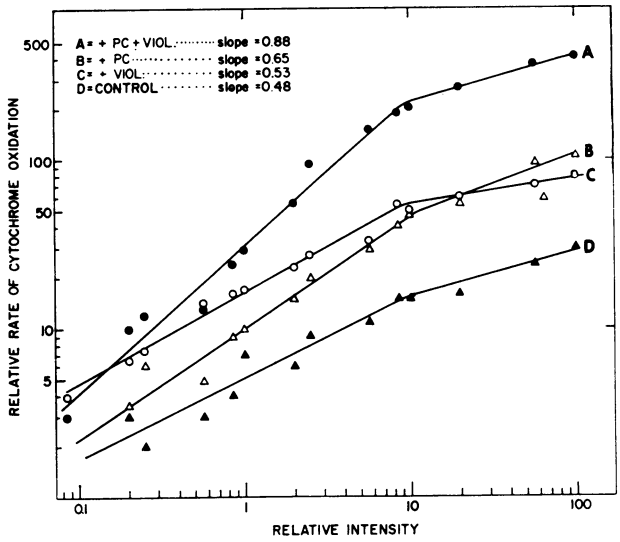

FIGURE 2

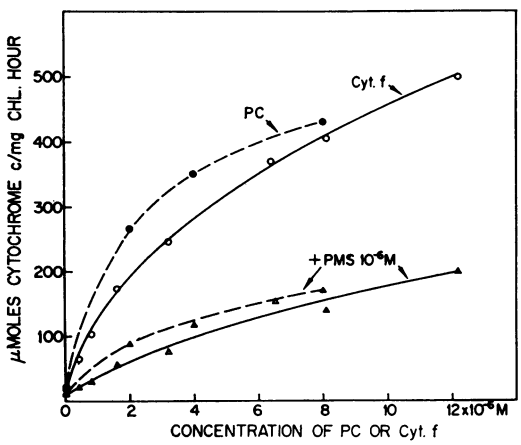

FIGURE 3

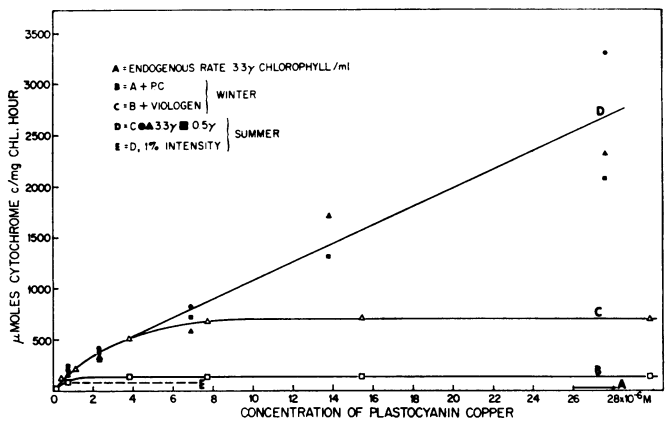

FIGURE 4

FIG. 1. Fraction of chlorophyll, P700, and cytochrome c photooxidation activity retained after treatment of chloroplasts with various concentrations of acetone.

FIG. 2. Rate of cytochrome c photooxidation as a function of intensity in the absence or presence of methyl viologen (5×10^{-5} M) and plastocyanin (3×10^{-7} M). Reaction mixture, Tris buffer pH 7.4, contained per milliliter: 5 μg chlorophyll, 1 mg cytochrome c, 0.1 μmole KCN. Slope of double logarithmic plots lower than one indicate a nonlinear relation between amount of light and rate even in very weak intensities.

FIG. 3. Inhibition of cytochrome c photooxidation by PMS. Reaction conditions as in figure 2. Degree of inhibition is the same in absence or presence of plastocyanin (dashed lines, filled symbols) or cytochrome f (full lines, open symbols).

FIG. 4. Effect of increasing concentrations of plastocyanin upon the rate of cytochrome c photooxidation. The reaction mixture in Tris buffer pH 7.8 contained per milliliter: 0.5 to 3.3 μg chloroplast chlorophyll, 0.1 μmole viologen, 0.1 μmole KCN, and 1 mg cytochrome c reduced by means of hydrogen and palladium. Illumination with intermittent light of high intensity.

of the system is enhanced (figs 2,4). The action of these autooxidizible low potential, single electron transfer agents might be conceived of as an acceleration of overall reaction III:

$$X^- + \text{viologen}_{ox} \rightarrow X + \text{viologen}_{red} \quad \text{IVa}$$

$$\text{viologen}_{red} + \tfrac{1}{4} O_2 \rightarrow \text{viologen}_{ox} + \tfrac{1}{2} H_2O \quad \text{IVb}$$

The dyes are reduced by photoproduct X^- and reoxidized by air so that less of X^- can enter back reaction Ib. This interpretation implies that detergent treated chloroplasts retain the ability to generate a strong photoreductant. (cf. also 13)

An agent which apparently acts in opposite fashion is PMS, which proved to be a potent inhibitor of cytochrome c photooxidation. Figure 3 shows that a concentration of 10^{-6} M yielded a uniform 50% inhibition of the rate catalyzed by various concentrations of plastocyanin and cytochrome f. The most likely interpretation of this inhibition is an acceleration of reaction Ib. PMS mediates an effective short circuit between X^- and $P700^+$, being reduced by the first and reoxidized by the second photoproduct.

The Role of Plastocyanin. The cofactor which Nieman et al. (16) and Bishop et al. (2) called a cytochrome c photooxidase was found to show several features of Katoh's copper-containing protein plastocyanin (8, 9). Tests in each purification step of the oxidized minus reduced difference spectrum around 597 mμ where plastocyanin absorbs strongly revealed that the intensity of this band was correlated with cytochrome c photooxidase activity. The purity index of the preparation used by us was 8.0 (8).

Figure 4 shows the effect of increasing concentrations of plastocyanin upon the rate of ferrocytochrome c oxidation in strong, rate-saturating light. Plastocyanin stimulated the endogenous rate up to tenfold. In the presence of 10^{-4} M viologen, however, stimulation was up to 150-fold.

The maximal rates of cytochrome c photooxidation catalyzed by plastocyanin were observed to vary considerably with the leaf material. Greenhouse-grown spinach yielded much lower rates during midwinter than during other seasons. In contrast, the individual chloroplast preparations, if stored at low temperatures, lost their activity only slowly in the course of several days. Curve C in figure 4 represents an experiment made in January, and reveals a finite maximum rate of ~700 μmoles/mg chlorophyll hour attained at a plastocyanin concentration of $\sim 5 \times 10^{-6}$ M which is about equivalent to the total chlorophyll in the suspension.

Curve D in figure 4 shows results obtained frequently with market spinach during spring and summer. The rate increases with increasing plastocyanin concentration and no ultimate limit is indicated.

In considering the absolute rate values in figures 4 and 5, it should be kept in mind that the measurements were made with intermittent actinic light which illuminated the sample for only 40% of the time. Appropriate checks showed 1.5 to twofold higher rates in continuous illumination. Therefore, the observed rates of 3000 μmoles/mg chlorophyll hour, in fact indicate rates in continuous light of 5000 μmoles/mg chlorophyll hr, or higher. These rates are severalfold in excess of regular Hill reaction velocities and also surpass the ones observed in complete photosynthesis and PMS mediated photophosphorylation (1).

Interestingly, plastocyanin stimulates the oxidation rates in very weak light, thus in effect raising the quantum yield of the system. In the absence of the catalyst, no true linearity between light and rate was observed, even in the weakest intensity range we were able to study. The double logarithmic plots in figure 2 show a slope approaching unity only in the presence of plastocyanin. The control experiment yielded a slope of about 0.5. Addition of viologen increased the rate at all intensities but failed to raise the slope, i.e., to make the process linear with intensity. These data suggest the interference of a higher order back reaction of the photoproducts. Plastocyanin might remove one of the photoproducts ($P700^+$) before it can undergo this back reaction and thus allow full utilization of the light over a wide range of intensities.

Earlier data (14) showed that photooxidation occurs most efficiently in light with wavelength >690 mμ. Additional absolute measurements such as illustrated in table I, revealed that with freshly made, optimal preparations the quantum yield in long wave light can approach unity. (1 hν per equivalent or 4 hν per O_2 consumed.) The efficiency drops to half this value or less at shorter wavelengths.

Table I. *Quantum Efficiency of Cytochrome c Photo-Oxidation at Three Wavelengths*

The reaction mixture consisted of Tris buffer pH 7.8, 15μg chlorophyll, 1.5 mg ferrocytochrome c, 10^{-4} M benzylviologen, 10^{-4} M KCN, and a saturating concentration of plastocyanin. The total volume was 1.5 ml. Each experiment consisted of a number of rate measurements in intensities varying between 0.01 and 1 μE/minute.

Wavelength	678 mμ	710 mμ	721 mμ
% fractional absorption	56.7	4.7	2.9
Quantum requirement	2.5	1.9	1.2

The effectiveness of the enzyme in quite low concentrations (in weak light, 1 molecule per ⩽20 chlorophyll molecules is saturating) and the extremely high rates obtainable suggest a specific site of action and a specific role in overall photosynthesis. This specificity is emphasized by the observation that the blue protein isolated from mung beans by Shichi and Hackett (20) was fully inactive. Likewise, polyphenol oxidase proved inactive.

Analogous to the interpretation of the viologen effect, it is suggested that plastocyanin catalyzes dark reaction II:

$$Cu^+PC + P700^+ \rightarrow Cu^{++}PC + P700 \quad \text{Va}$$

$$Cu^{++}PC + Fe^{++}\text{cyt c} \rightarrow Cu^+PC + Fe^{+++}\text{cyt c} \quad \text{Vb}$$

This interpretation is supported by the observations that ferrocytochrome c reduces oxidized plastocyanin instantaneously and that detergent treated chloroplasts in the presence of viologen photooxidize reduced plastocyanin with an initial rate comparable to that of the catalyzed cytochrome c photooxidation (400–800 μeqs/chlorophyll hour).

The Role of Cytochrome f. Cytochrome f, prepared from *Euglena gracilis* (OD 280/OD 415 = 0.2, normal potential 0.37 v, α band 552 mμ) (11) was found to be a catalyst for cytochrome c photooxidation. Like plastocyanin, it enhances the rate in weak light, i.e., increases the quantum yield of the process considerably (fig 5). Figure 5 also shows

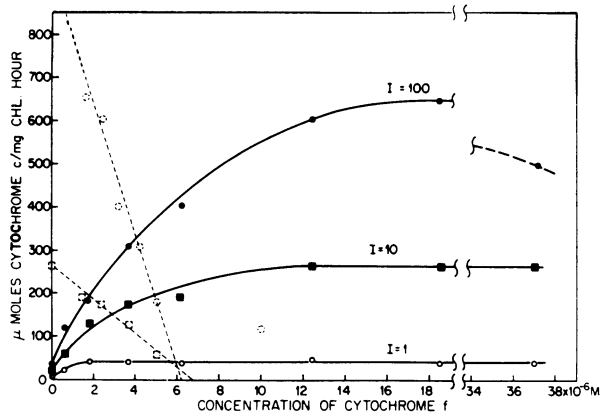

FIGURE 5

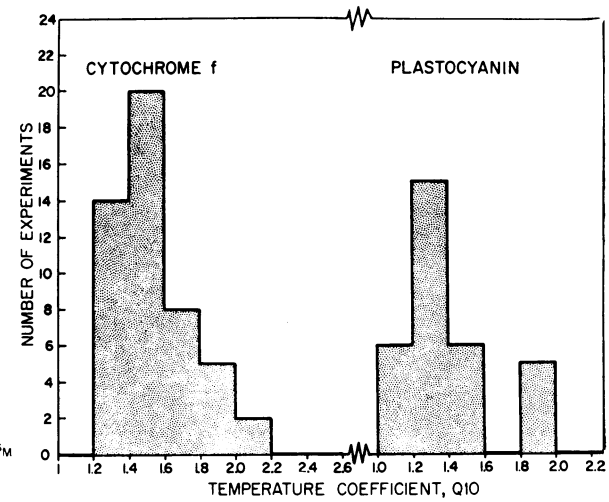

FIGURE 6

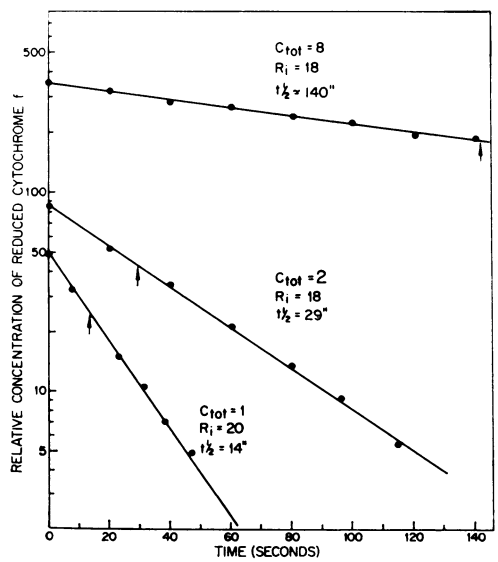

FIGURE 7

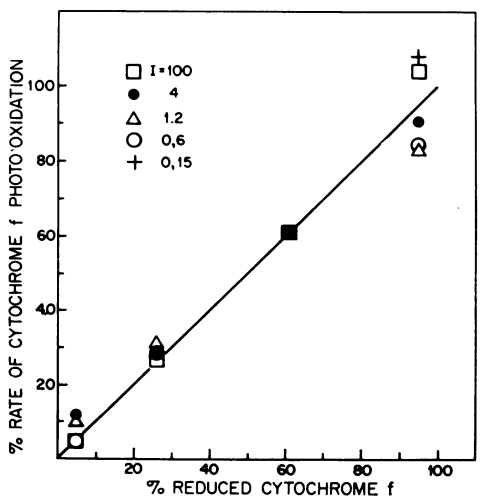

FIGURE 8

FIG. 5. Effect of various concentrations of cytochrome f upon the rate of cytochrome c photooxidation in 3 light intensities. Reaction conditions as in figure 4. Dotted slopes and symbols are replots (Y vs. Y/X) of the corresponding full curves and indicate hyperbolic shape of the latter.

FIG. 6. Frequency distribution of the temperature coefficient Q_{10} observed for the photooxidation of cytochrome c catalyzed by cytochrome f and by plastocyanin. (Experimental conditions cf legend figure 4).

FIG. 7. Time course of the photooxidation of cytochrome f observed with 3 concentrations (C_{tot}) of the substrate. R_i denotes the initial reaction rate. Reaction conditions as described in legend of figure 4 except for the replacement of cytochrome c by cytochrome f.

FIG. 8. Dependence of the rate of cytochrome f photooxidation upon the fraction of the substrate which is in the reduced form. Measurements were made in 5 intensities (I=20% was saturating). Data, obtained as described in text, were normalized by assuming in each intensity the 62% reduced substrate yielded 62% of the maximal rate.

that the rates in high light intensities are stimulated (~25-fold) and that an increasing amount of enzyme is required for saturation in higher light intensities. The dotted curves in figure 5 are replots of the 100 % and 10 % intensity curves showing rate vs. rate/cytochrome f concentration (7). These plots intercept the abscissa at a value of 0.12 sec^{-1}, reveal a hyperbolic function, and a rate of ~1000 eqs/chlorophyll hour at saturating enzyme concentration and intensity.

In contrast to the situation found with plastocyanin as a catalyst, the system stimulated by cytochrome f in strong light always attained a limited maximum rate. This maximum velocity was affected only slightly by seasonal changes in the spinach and varied between 500 and 700 μeqs/chlorophyll hour (i.e., between 1000 and 1500 after correction for the intermittency effect of the illumination procedure).

The catalytic effect of cytochrome f can be considered as entirely analogous to that of plastocyanin (cf Va and Vb). Its oxidized form also reacts rapidly with reduced cytochrome c.

Effect of Temperature. Bishop et al. (2) reported a surprisingly low activitation energy of 4.3 kcal/mole for the photooxidation of cytochrome c even in saturating light intensities. However, the maximal rates attained in their study were relatively low (~30 μeqs/chlorophyll hour) and we therefore reinvestigated the effect of temperature under conditions of saturating light, viologen, and plastocyanin or cytochrome f.

Our experiments covered a span between 25 and −5° and were made with various catalyst concentrations and chloroplast preparations. To avoid freezing, 10 % methanol was added to the samples after it was found that this addition does not affect the rates of photooxidation. In some experiments the temperature was changed by 10° intervals, in other cases Q_{10} was computed as the cube root of the ratio between 2 rates, measured at 25° and −5°. Figure 6 shows the frequency distribution of all the data. They indicate an activation energy of 6 kcal/mole for the cytochrome f catalyzed system and a value of 4.3 kcal/mole for the plastocyanin catalyzed reaction.

The actinic light routinely used in this study was interrupted by a rotating disc, the light period being 6.8 milliseconds and the dark time 9.8 milliseconds. Without any effect of the dark periods, a rate of 40 units with the disc revolving would be expected if continuous saturating light gave a rate of 100 units. In experiments designed to check this point, we observed 55 to 60 relative rate units (plastocyanin catalysis, rates 500 to 700 μeqs/cholorophyll hour) which shows that during the dark time a preparatory or finishing dark reaction occurs which enhances the overall rate (up to 50 %). As was to be expected, we found the temperature coefficient to be negligible for this intermittency factor and identical for continuous and intermittent light.

The low temperature characteristic, especially of the plastocyanin system, (rates up to 2000 μmoles/mg chlorophyll hour were observed at −5°) is quite different from the more normal Q_{10} values of 2 to 3 reported for the saturation rate of intact cell photosynthesis (19), the Hill reaction (15), and PMS mediated photophosphorylation (6). A maximal velocity which is only slightly dependent upon temperature could be due to one of several mechanisms: the net rate might be a balance between a forward and a back process having only slightly different temperature coefficients or, the limiting step could be the charge transfer from cytochrome f to $P700^+$. Witt et al. (21) reported that this step occurs even at liquid nitrogen temperature and requires 3 to 5 milliseconds at temperatures between O° and 40° (cf. also 3).

Kinetics of Cytochrome f Photooxidation. Ferrocytochrome f used in substrate amounts in place of cytochrome c was photooxidized with high efficiency and rate. Viologen in 10^{-4} M concentration enhanced the rates in strong light from 150 to 550 μeqs/chlorophyll hour. However, unlike the case with cytochrome c, plastocyanin (5×10^{-6} M) did not stimulate the photooxidation of cytochrome f.

In the presence of adequate catalysts, ferrocytochrome c is photooxidized at a practically constant rate until near depletion. This course is similar to the zero-order time course of substrate reduction in photosynthesis and most Hill reactions.

Cytochrome f photooxidation, in contrast, follows very different and rather unexpected kinetics. The lower curve in figure 7 shows the photoconversion of a small amount of cytochrome f, 65 % of which was initially in the reduced form. The reaction appears to follow an exponential time course, with a half-time of 14 seconds. After some 60 seconds exposure, oxidation was complete and addition of ferricyanide showed no further effect.

In a second experiment (middle curve, fig 7) with a twofold higher concentration of cytochrome f, an identical initial rate was obtained as in the previous case. Again, the time course appeared to be first order, but the half-time was twofold greater than in the first experiment. The top curve in figure 7 shows that an eightfold higher concentration again yields the same initial rate but a tenfold greater half-time. With high concentrations of cytochrome f it takes very long exposures to approach complete oxidation and evaluation of the time course becomes difficult. Also, secondary inactivation effects interfere in strong light.

The data suggest that in saturating light, the rate is independent of substrate concentration, but proportional to the ratio reduced cytochrome f/total cytochrome f:

$$\text{Rate} = k\ \text{Cyt}\ f_{red}/\text{Cyt}\ f_{total} \qquad \text{VI}$$

In similar experiments, the initial rate of photooxidation was measured as a function of the redox state of the cytochrome, or after adding successive amounts of substrate. Reasonable agreement with the above rate equation was found.

Interestingly enough, not only the saturation rate

but also the quantum yield of cytochrome f photooxidation is determined by this ratio. The data of figure 8, which illustrate this, were obtained in the following way: cytochrome f, 95 % of which was in the reduced state was added to a viologen containing chloroplast suspension. To determine the relative quantum yield, the rate of photooxidation was measured in 4 weak intensities of red light. Subsequently, the sample was exposed to strong (100 %) light, which yielded the saturation rate and left 62 % of the cytochrome f in the reduced state. Again we measured 4 rates in weak intensities. This sequence of exposure was repeated 4 times. The results indicate that at all intensities the rate is proportional to the per cent reduction of the substrate. Assuming a hyperbolic shape for the rate versus intensity curve, which seems to be justifiable, the general equation VII applies:

$$\text{Rate} = -\text{dcyt } f_{red}/dt = \text{cyt } f_{red}/\text{cyt } f_{tot}\ [k\Phi I/k + \Phi I] \quad \text{VII}$$

in which Φ represents the quantum yield and k the rate limiting (dark) reaction constant.

High light intensities:

$$R_{max} = k \text{ cyt } f_{red}/\text{cyt } f_{total} \quad \text{VIIa}$$

Low light intensities:

$$R = \Phi I \text{ cyt } f_{red}/\text{cyt } f_{total} \quad \text{VIIb}$$

The high rates and yields and the zero-order time course of cytochrome f catalyzed cytochrome c photooxidation are now easily explainable: as long as a small amount of ferrocytochrome c is present, all cytochrome f will be in the reduced form, which allows maximal rates and yields.

Discussion

Mediation by Particle Bound Cytochrome f. A chloroplast suspension contains a number of photosynthetic units. Each unit is equipped with a few hundred sensitizing chlorophyll molecules from which absorbed quanta flow to its reaction center which contains one molecule each of P700, X, cytochrome f, and plastocyanin. Only in case such a center is in the appropriate condition can conversion of a light quantum take place; otherwise, it is lost. That is, P700 has to be in the reduced (colored) form and X in the oxidized state. If only n % of all photosynthetic units in the suspension are in this favorable condition, the quantum efficiency will be n % of the maximal value. That the redox state of X determines the probability of a successful photochemical reaction might partly explain the stimulatory effect of viologen upon the quantum yield. Acceleration of the reoxidation of X_{red} (besides reducing the rate of back reaction Ib), aids in reestablishing initial conditions.

$P700^+$, on the other hand, is reduced by cytochrome f. If a simple collision process were involved, the quantum yield would depend only upon the concentration of the reduced cytochrome regardless of the presence of the oxidized form. However, the data presented in the previous section (fig 8) showed that the chance of a quantum performing photochemistry is determined by the fraction of the exogenous cytochrome f present in the reduced state (VIIb). To explain this, we must assume that exogenous cytochrome f equilibrates with a fixed amount of cytochrome f intimately associated with the photoconverters. The fraction of this bound material which is in the reduced state then determines the chance of a photoact being effective. Why need the cytochrome f be reduced for photochemistry to occur in a conversion center? This intermediate is not itself the primary receptor, but only serves to donate an electron to photooxidized P700. The normal potential of P700 is a good deal higher than that of cytochrome f so that when half of the cytochrome f was in the reduced state at equilibrium, P700 would be about completely reduced and the quantum yield should be nearly optimal. Actually, however, only half the optimal yield is found. Obviously the photoact is ineffective unless both cytochrome f and P700 are in the reduced state. Thus the two components form an intimate complex and an electron, photochemically transferred from P700 to X will return from X^- to $P700^+$, unless an electron from cytochrome f^- fills the "hole" immediately.

In preparations enriched in P700 by means of acetone extraction, P700 can be (photo) oxidized and reduced without the mediation of cytochrome f (10), and $P700^+$ is quite stable. Therefore, there seems no need to assume that the reduced state of bound cytochrome f is required prior to or during the occurrence of the primary photoact itself.

Significance of the Observations for Photosynthesis. The data presented support the validity of reaction sequence I to III. Accordingly, P700 collects quanta from its surrounding chlorophyll a molecules, which constitute the long-wave photosystem of photosynthesis. Excitation results in the transfer of an electron to a reaction partner, X, which then becomes a strong reductant, able to reduce viologen dye. $P700^+$ regains an electron from or via plastocyanin or cytochrome f. Both components appear to be specific electron donors for the primary photooxidant occupying specific sites on the chloroplast matrix. As will be described elsewhere, the kinetics of the photooxidation of plastocyanin in substrate amounts are very similar to that of cytochrome f. It is somewhat disappointing that despite the relative simplicity of the photooxidation process, we could not conclusively localize the rate-limiting step in the reaction chain. Future experiments will have to decide whether a finite limiting rate, independent of light and very little dependent upon temperature, is imposed by the charge transfer from cytochrome f or plastocyanin to $P700^+$, by a conformational change of the matrix, or a competing back reaction between photoproducts.

Under conditions in which the redox state of particle bound cytochrome f (or plastocyanin) deter-

mines both the efficiency and maximum rate, the system shows features resembling complete photosynthesis. One may assume that in the complete process, ferrocytochrome f or cupro plastocyanin are generated by the second photoreaction. If the rate of this reduction cannot keep pace with the rate of P700 photooxidation, the overall process will run with a poor quantum yield. This is observed in long wavelength light where fewer quanta excite the second photoact than the first photoact.

One could speculate that in whole cells and fresh chloroplasts reduction of $P700^+$ occurs either by PC or cytochrome f, the two pathways possibly yielding different products of photosynthesis. Indeed some observations (4, 11) did indicate that cytochrome f mediates only part of the electron transport in photosynthesis.

An alternate speculation, which also accounts for these observations, was forwarded elsewhere (11): the possibility was considered that in fresh chloroplasts, PC and cytochrome f function in parallel in the reduction of $P700^+$, and the first two components or a complex of them play a direct role in the second, O_2 evolving photoact. This speculation was prompted by the analogy of the cytochrome c photooxidation system with the respiratory cytochrome c oxidase system, in which also a copper and an iron atom operate in close conjunction (5). It might therefore be a fruitful working hypothesis to conceive the O_2 evolving photoreaction of photosynthesis, in essence, as a reversal of the terminal respiration step driven by light and sensitized by chlorophyll.

Summary

An analysis was made of the long wavelength sensitized photooxidation by detergent treated spinach chloroplasts of ferrocytochrome c, ferrocytochrome f, and cupro plastocyanin. All systems were stimulated (up to sixfold) by viologen dyes and other autooxidizable, low potential, single electron transfer agents. In the presence of viologen, the photooxidation of ferrocytochrome c was stimulated by catalytic amounts of cytochrome f and plastocyanin. These agents respectively stimulated up to 30- and 150-fold and sustained rates up to 1500 and 5000 μmoles/mg chlorophyll hour. In all cases, the quantum yield as well as the light saturated rate was enhanced. Quantum yields approaching unity were observed in long wave red light. The light saturated rate especially of the plastocynanin catalyzed system is only slightly dependent upon temperature and rates up to 2000 eq/chlorophyll hour were observed below 0°. In the presence of viologen, cupro plastocyanin and ferrocytochrome f are themselves photooxidized with high efficiency and rate. Particle bound cytochrome f mediates the reaction and its redox state in a charge transfer complex with P700 determines the probability of a photoact being successful. The data are interpreted on the assumption that the first long-wave photoaction of photosynthesis is operative in this system. A speculation is forwarded concerning the possible role of the two heavy metal catalysts in the second photoact of complete photosynthesis.

Acknowledgments

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Literature Cited

1. Avron, M. 1960. Photophosphorylation by Swiss Chard chloroplasts. Biochim. Biophys. Acta 40: 257–72.
2. Bishop, N. I., H. Nakamura, J. Blatt, and B. Vennesland. 1959. Kinetics and properties of cytochrome c photooxidase of spinach. Plant Physiol. 34: 551–58.
3. Chance, B., and M. Nishimura. 1960. On the mechanism of chlorophyll-cytochrome interaction: The temperature insensitivity of light-induced cytochrome oxidation in chromatium. Nat. Acad. Sci. 46: 19–24.
4. Duysens, L. N. M. and J. Amesz. 1962. Function and identification of two photochemical systems in photosynthesis. Biochim. Biophys. Acta 64: 243–60.
5. Griffiths, D. E. and D. C. Wharton. 1961. Studies of the electron transport system. Purification and properties of cytochrome oxidase. J. Biol. Chem. 236: 1850–56.
6. Hall, D. O. and D. I. Arnon. 1962. Photosynthetic phosphorylation above and below 0°. Proc. Natl. Acad. Sci. 48: 833–39.
7. Hofstee, B. H. J. 1952. On the evaluation of the constants *Vm* and *Km* in enzyme reactions. Science 116: 329–31.
8. Katoh, S. 1960. A new copper protein from *Chlorella ellipsoidea*. Nature 186: 533–34.
9. Katoh, S., I. Shiratori, and A. Takamiya. 1962. Purification and some properties of spinach plastocyanin. J. Biochem. (Tokyo) 51: 32–40.
10. Kok, B. 1961. Partial purification and determination of oxidation reduction potential of the photosynthetic chlorophyll complex absorbing at 700 mμ. Biochim. Biophys. Acta 48: 527–33.
11. Kok, B., B. Cooper, and L. Yang. 1963. Electron transport in chloroplast reactions, p. 373–96. In: Microalgae and Photosynthetic Bacteria. Univ. Tokyo Press.
12. Kok, B. and G. Hoch. 1961. Spectral changes in photosynthesis. In: A Symposium on Light and Life, W. D. McElroy, ed. Johns Hopkins Univ. Press, Baltimore.
13. Kok, B., and G. Hoch. 1963. The photoreactions of photosynthesis. In: Proc. Colloq. Photosynthese. Paris. p. 93–107.
14. Kok, B., G. Hoch, and B. Cooper. 1963. Sensitization of chloroplast reactions. I. Sensitization of reduction and oxidation of cytochrome c by chloroplasts. Plant Physiol. 38: 274–79.
15. Lumry, R. and J. D. Spikes. 1962. Chemical kinetic studies of the Hill reaction, p. 373–91. In: Research in Photosynthesis, H. Gaffron, ed. Interscience, New York.

16. NIEMAN, R. H., H. NAKAMURA, AND B. VENNESLAND. 1959. Fractionation and purification of cytochrome c photooxidase of spinach. Plant Physiol. 34: 262–64.
17. NIEMAN, R. H., AND B. VENNESLAND. 1959. Photoreduction and photooxidation of cytochrome c by spinach chloroplast preparations. Plant Physiol. 34: 255–62.
18. NISHIMURA, M. 1959. New hematin compound isolated from *Euglena gracilis*. J. Biochem. (Tokyo) 46: 219–23.
19. RABINOWITCH, E. I. 1945. Photosynthesis. I. Interscience, New York.
20. SHICHI, H., AND D. P. HACKETT. 1963. Purification and properties of a blue protein from etiolated mung bean seedlings. Arch. Biochem. Biophys. 100: 185–91.
21. WITT, H. T., A. MÜLLER, AND B. RUMBERG. 1963. Electron-transport system in photosynthesis of green plants analyzed by sensitive flash photometry. Nature 197: 987–91.

ATP-Induced Inhibition of Potato Browning. Effect of Ascorbic Acid Oxidase and of Reducing Substances [1]

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When potato tubers and some other plants are sliced, polyphenol oxidase catalyses the oxidation of phenols to form dark polymers. Reducing substances, such as ascorbic acid, prevent this browning by reducing the quinones formed in diphenol oxidation (6, 8). Browning of potato slices begins only after all ascorbic acid has been oxidized.

Previous studies have shown that plant slices treated with solutions of ATP remain substantially lighter than untreated controls (5). The interference of ATP with enzymic color formation appears to depend on metabolically active particulate systems. With purified polyphenol oxidase enzyme or particle-free extracts, ATP has no effect on the course or the products of the polyphenol oxidase reaction (4). Although ATP itself is not a reducing substance, its effect on plant slices resembles the effect of ascorbic acid or other reducing substances.

A possible explanation of the effect of ATP on potato slices is the activation of a mechanism for reduction of dehydroascorbic acid, or reduction of oxidized glutathione, or both. These reductions are closely interrelated inasmuch as the enzymic reduction of dehydroascorbic acid requires glutathione (6, 7).

If a reductive formation of ascorbic acid or glutathione were responsible for inhibition of browning by ATP, then this inhibition should be enhanced by adding the precursors, oxidized glutathione or dehydroascorbic acid, to slices treated with ATP. Furthermore, the prevention of browning by ATP should be nullified on addition of ascorbic acid oxidase. It will be shown that ascorbic acid does not appear to be the reducing substance formed upon addition of ATP. The relative effectiveness of several reducing compounds on enzymic browning of potato slices will also be given.

Materials and Methods

Russet and White Rose potatoes (*Solanum tuberosum*, L.) were stored at 5° before use. Methods and experimental conditions were similar to those used earlier (5). Substrate 3,4-DL-dihydroxyphenylalanine was added to all samples including controls. Essentially the same results were obtained with and without added substrate but the rate of browning was slower without it. All materials and solutions were kept at 1 to 2°. Polyphenol oxidase was purchased from Mann Biochemicals.[2] Crude preparations of ascorbic acid oxidase were made from cucumber and from summer squash (1); a highly purified preparation was a gift from C. R. Dawson. Dehydroascorbic acid was from Nutritional Biochemicals Company and was further purified by precipitation at 0° with absolute ethanol (3). Crystalline disodium salt of ATP was purchased from Pabst Laboratories.

Color changes were measured by reflectance directly on 6 mm thick potato slices and read on the Rd scale of a Gardner Automatic Color Difference Meter. Two slices with matched opposing surfaces from a single cut served as control and treatment

[1] Received Sept. 20, 1963.

[2] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.