Flash Spectrophotometric Studies of Chlorophyll-sensitized Oxidation-Reduction Reactions*

Bacon Ke, Leo P. Vernon, and Elwood R. Shaw

ABSTRACT: When an anaerobic solution of chlorophyll *a* in ethanol was illuminated by a 20-μsec red actinic flash, transient-absorption decrease and increase were observed at 430 and 470 mμ, respectively. The difference spectrum agrees closely with that calculated from the reported singlet and triplet absorption spectra of chlorophyll *a*. Various oxidants such as pyocyanine, phenazine methosulfate, ubiquinone-6, and trimethylquinone (but not cytochrome *c*) modify the decay kinetics of the transient absorption change of chlorophyll *a*. The difference spectra remain the same as that of chlorophyll *a* alone. The data suggest that the photoexcited chlorophyll rapidly transfers an electron to the oxidant molecule, forming a positive radical ion of chlorophyll and a semiquinone. The latter species then back-react in the dark. A complex absorption-change transient consisting of both rapidly and slowly decaying portions was observed at 430 mμ when the system chlorophyll–pyocyanine–reduced ubiquinone-6 in slightly acidic ethanol was illuminated. Evidence from the difference spectra, the effect of the concentration of reduced quinone on the decay kinetics, and the rapid rise-time of the absorption change owing to pyocyanine reduction indicate that chlorophyll excitation was followed by an instantaneous transfer of an electron from chlorophyll to pyocyanine. The positive radical ion of chlorophyll then reacts with the reduced quinone. In the system chlorophyll–cytochrome *c* (oxidized)–trimethylquinone (reduced) in ethanol, evidence from the difference spectra, the slow rise-time of the absorption-change transient due to cytochrome, and the effect of the initial concentration ratio between oxidized and reduced trimethylquinones on the decay kinetics suggest that the initial electron transfer from the excited chlorophyll was to the oxidized quinone and that reduction of cytochrome occurred in the dark.

Oxidation-reduction reactions sensitized by chlorophyll in solutions have been studied extensively, mainly because of important bearings these *in vitro* reactions might have on the mechanism of the primary act in photosynthesis (Livingston, 1960; Krasnovsky, 1961). One of the most interesting reactions is the so-called Krasnovsky reaction, which involves the reversible photoreduction of chlorophyll by ascorbate (Krasnovsky, 1948). Most chlorophyll-sensitized reactions involve either an oxidation of a substrate (e.g., allylthiourea) by molecular oxygen or the transfer of hydrogen from some reducing agent (e.g., ascorbic acid) to some oxidized substance (e.g., methyl red). Biochemically significant and interesting extensions from these chlorophyll-sensitized redox reactions have been made to the photoreduction of cytochrome *c* in either ethanol or aqueous phosphate buffer (Krasnovsky, 1955; Vernon, 1961a) and to the photoreduction of pyridine nucleotide in aqueous media (Vernon, 1961b).

It has recently been shown that chlorophyll from either bacterial chromatophores or plant chloroplasts catalyzes reactions involving redox agents with important functions in actual photosynthesis. Zaugg (1963; Zaugg *et al.*, 1964) reported the photooxidation of reduced cytochrome *c* or reduced PMS[1] coupled to the photoreduction of ubiquinone by bacterial chromatophore fragments. Although an aerobic photooxidation of reduced cytochrome *c* was reported earlier for aged chloroplasts (Nieman and Vennesland, 1959), coupling of this reaction with quinone reduction has not yet been demonstrated for plant chloroplasts. It has been found, however, that an oxidation of reduced PMS coupled to the reduction of ubiquinone occurs with plant chloroplasts (Vernon *et al.*, 1963). Preliminary studies (Vernon *et al.*, 1963, 1964) showed that similar coupled redox reactions involving PMS can also be sensitized by isolated chlorophyll in an organic solvent such as ethanol.

Usually chlorophyll-sensitized reactions are studied by following the time course of absorption changes of one of the reacting species upon illumination. More recently, electron paramagnetic resonance (EPR) has also been used to study light-induced reactions between electronically excited chlorophyll and various quinones or other donor-acceptor couples (Tollin and Green, 1962, 1963).

In chlorophyll-sensitized reactions the manner of participation by chlorophyll is still largely a matter of

* From the Charles F. Kettering Research Laboratory, Yellow Springs, Ohio. *Received August 7, 1964.* Contribution No. 163 from the Charles F. Kettering Research Laboratory.

[1] Abbreviations used in this work: PMS, phenazine methosulfate; TMQ, $TMQH_2$, oxidized and reduced trimethyl-*p*-benzoquinone, respectively; UQ_6, UQ_6H_2, oxidized and reduced ubiquinone with six isoprene units in the side chain; Cyt *c*, highly purified "type III" cytochrome *c;* Pyc, pyocyanine; Chl, chlorophyll; EPR, electron paramagnetic resonance.

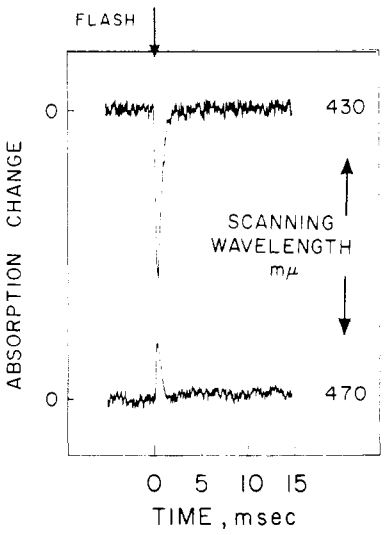

FIGURE 1: Absorption-change transients of chlorophyll *a* at 430 and 470 mμ. Chlorophyll concentration. 20 μg/3 ml ethanol; excitation wavelength, 640–740 mμ; flash duration, 20 μsec.

speculation. The chlorophyll reaction is so fast that it usually cannot be observed by conventional means. Although flash photolysis has been used for observing the triplet state of chlorophyll formed by intense illumination (Livingston, 1955; Linschitz and Sarkanen, 1958; Claesson *et al.*, 1959), it was not until very recently that the rapid technique of flash photometry was applied to the observation of transient-absorption changes of chlorophyll in the Krasnovsky reaction (Zieger and Witt, 1961).

The present paper reports some observations made on coupled redox reactions between phenazine dyes or cytochrome *c* and several quinones sensitized by chlorophyll *a* in ethanol. The rapid technique of flash spectrophotometry was used mainly for examining the fast chlorophyll reactions that ordinarily are not observable by conventional techniques.

Experimental

The construction of the flashing-light spectrophotometer and operating procedures have been described recently (Ke *et al.*, 1964). A CAT-computer (Mnemotron Corp., White Plains, N.Y.) was used to improve the signal-to-noise ratio. Broad-band red (640–740 mμ) flashes with 20-μsec duration and an intensity of 7.8×10^{14} quanta·cm^{-2} per flash were used for illumination. In most of the experiments fifty repetitive flashes spaced at 2-second dark intervals were used to obtain the final signal. All absorption-change transients presented in this paper represent transcriptions of the CAT-computer signal onto an *xy*-recorder. Steady-state absorption changes caused by continuous illumination were measured on a modified Beckman Model DB recording spectrophotometer (Zaugg, 1963).

Chlorophyll *a* was prepared from spinach leaves and separated on a sugar column according to the procedure of Zscheile and Comar (1941). A stock solution of chlorophyll was prepared in ether. PMS and TMQ or $TMQH_2$ were obtained from K and K Laboratories (Jamaica, N.Y.) and were used without purification. UQ_6 and a highly purified "type III" cytochrome *c* (Cyt *c*) were purchased from Sigma Chemical Co. (St. Louis, Mo.). The UQ_6 was reduced by dithionite, extracted, and redissolved in acidic ethanol (Green and Burkhard, 1961). Pyocyanine was prepared by exposing an aerobic solution of PMS to sunlight at *p*H 8 for several hours. Crystalline pyocyanine was dissolved in an aqueous solution 0.1 M in HCl. Consequently, the ethanolic system containing pyocyanine as a component became slightly acidic and had an apparent *p*H of 2.6. However, the absorption spectrum of chlorophyll remained unchanged, indicating the chlorophyll was not converted into pheophytin under the conditions used. All reactions were carried out in ethanol under anaerobic conditions (Vernon, 1963) unless otherwise stated.

Results

Formation of Chlorophyll a Triplet State in Ethanol. The relatively long-lived state of activated chlorophyll can be formed by intense flash illumination. Triplet-state chlorophyll and related compounds show absorption bands which are broad and relatively structureless. The major absorption band of the triplet state of chlorophyll *a* occurs slightly to the longer-wavelength side of the blue band of the singlet state (Livingston, 1955; Linschitz and Sarkanen, 1958; Claesson *et al.*, 1959). Depending on the wavelength of the measuring beam, the transient-absorption change observed with the flashing-light spectrophotometer could be either a decrease or an increase. Figure 1 shows transient-absorption changes at 430 and 470 mμ brought about by 20-μsec red actinic flashes. The half-life of the excited state was estimated from the expanded time scale of the CAT-computer to be 520 ± 40 μsec, well within the range reported for chlorophyll *a* in some other solvents (Livingston and Ryan, 1953). With less thorough evacuation, a shorter lifetime of 270 μsec was observed. Presumably, in the latter case, a trace of residual oxygen acted as a quencher for the triplet state and caused a more rapid decay. Slight exposure of the anaerobic system to the atmosphere completely eliminated the signals. The difference spectrum obtained by varying the scanning wavelengths agrees very closely with that calculated from the singlet and triplet spectra reported in the literature (Livingston, 1955; Linschitz and Sarkanen, 1958; Claesson *et al.*, 1959). The isosbestic point occurred near 450 mμ.

Chlorophyll-sensitized Reaction for a Single Oxidant. With an oxidant present in the chlorophyll solution and in the absence of oxygen, flash illumination usually induces a rapid absorption decrease at 430 mμ, followed by a decay whose rate depends on the nature of the oxidant. Figure 2 shows the transient-absorption change at

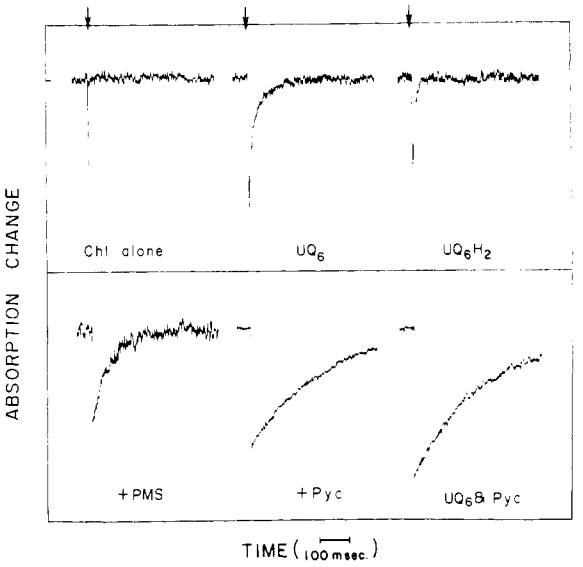

FIGURE 2: Absorption-change transients of ethanolic solutions containing chlorophyll *a* alone or with one (or two) oxidants. Concentrations used: chlorophyll, 20 μg/3 ml solution; oxidant, approximately 0.2 μmole/3 ml solution.

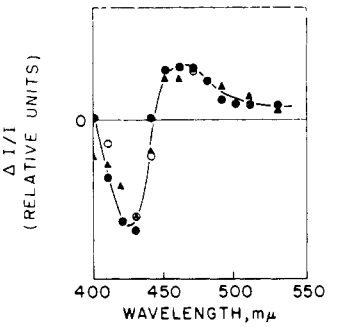

FIGURE 3: Difference spectrum of the rapid initial absorption changes in ethanolic solutions containing chlorophyll *a* plus pyocyanine (filled circles), PMS (open circles), or UQ_6H_2 (triangles). Solution composition same as in Figure 2.

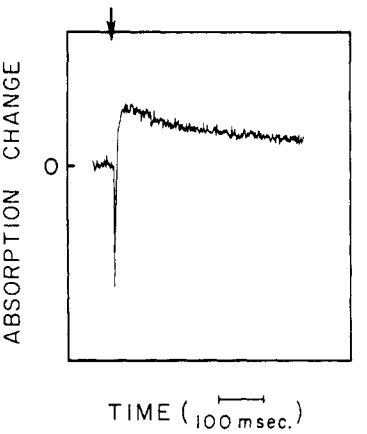

FIGURE 4: Absorption-change transient at 430 mμ for the system Chl-Pyc-UQ_6H_2-ethanol. Concentrations used (in 3 ml solution): chlorophyll, 20 μg; pyocyanine, 0.2 μmole; UQ_6H_2, 0.2 μmole. Apparent *p*H of the ethanolic solution, 2.6 (acidity owing to HCl contained in the pyocyanine solution).

430 mμ of an anaerobic solution of chlorophyll plus one of several oxidants. The rate of the initial absorption change of the mixed solution was as rapid as that of the chlorophyll solution alone. However, in the presence of an oxidant, the decay time was always longer. The decay of the absorption change probably represents the back-reaction of the products formed in the initial photochemical reaction. Slight exposure of the anaerobic system to the air completely eliminated the signal.

The chlorophyll solution containing UQ_6H_2 yielded a similar rapid initial decrease followed by a more rapid decay. Since reduced quinone invariably contained a small percentage of oxidized species, it is suspected that the reaction may actually have been caused by the oxidized quinone present in the solution. The more rapid decay, or a more rapid back-reaction, may also be explained as caused by the excess reduced quinone present. The absorption-change transient at the lower right of Figure 2 shows an interesting case where the chlorophyll solution contained two oxidized species, namely, UQ_6 and pyocyanine. The decay kinetics is almost identical with that of pyocyanine, indicating the latter reacts preferentially with the activated chlorophyll. TMQ gave a decay constant of 5 msec (transient not shown in Figure 2), showing the most rapid decay among all the oxidants examined. The decay of the transient-absorption change of chlorophyll was altered by all the oxidants studied in this work except cytochrome *c*.

The difference spectra of the chlorophyll-sensitized reactions containing one oxidant followed very closely the profile of the difference spectrum of the chlorophyll triplet state. Normalized values of the absorption changes for chlorophyll with either PMS, pyocyanine, or UQ_6H_2 are shown in Figure 3.

Chlorophyll-sensitized Redox Reactions. CHL-PYC-UQ_6H_2-ETHANOL SYSTEM. In the ethanolic chlorophyll solution containing pyocyanine or PMS plus either UQ_6H_2 or $TMQH_2$, a chemical reduction of PMS or pyocyanine by the reduced quinone occurred before illumination commenced (Vernon *et al.*, 1963, 1964). Thus oxidized and reduced species of both compounds were present in an equilibrium mixture prior to illumination. Upon flash illumination, a rapid absorption decrease took place at 430 mμ. The initial absorption decrease recovered rapidly; the decay constant was approximately 2 msec. Subsequently a slightly slower absorption increase occurred and was followed by a slow decay. A typical transient absorption change at

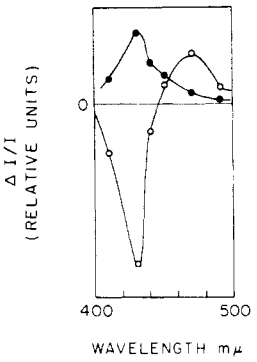

FIGURE 5: Difference spectrum of the system Chl-Pyc-UQ_6H_2-ethanol. The rapidly decaying portion (open circles) and the slowly decaying portion (filled circles) are plotted separately.

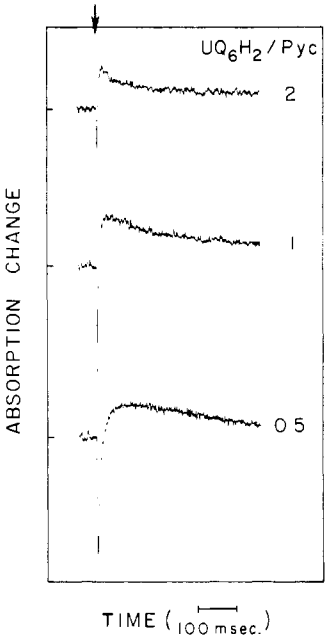

FIGURE 6: Effect of reduced ubiquinone concentration on the absorption-change transients in the system Chl-Pyc-UQ_6H_2-ethanol. Concentrations used (in 3 ml solution): chlorophyll, 20 μg; pyocyanine, 0.2 μmole; UQ_6H_2, 0.4, 0.2, and 0.1 μmole, respectively.

430 mμ for the complete system containing chlorophyll and the redox couple is shown in Figure 4.

Difference spectra of the rapid and slow portions of the transient signal, as shown in Figure 5, indicate that the rapid absorption changes with a negative peak at 430 mμ and a positive peak near 470 mμ are probably attributable to the formation of an electronically excited state of chlorophyll, whose profile is very similar to that of the chlorophyll triplet state. The difference spectrum of the slow portion with a positive peak at 434 mμ may be assigned to a stable semiquinone of

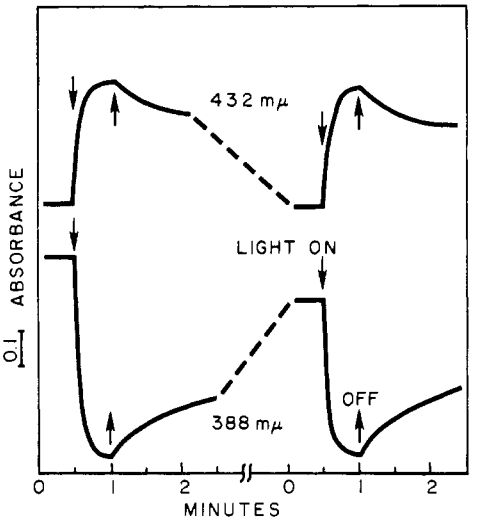

FIGURE 7: Absorption changes in the system Chl-Pyc-UQ_6H_2-ethanol with continuous illumination. Solution composition same as in Figure 4.

pyocyanine. In a recent study (Zaugg, 1964), PMS and pyocyanine were found to produce stable semiquinones in acid solution, with absorption peaks at 458 and 436 mμ, respectively.

When the concentration of UQ_6H_2 was varied (with the pyocyanine concentration held constant), the recovery rate of the initial rapid absorption decrease also varied. As shown in Figure 6, at three different ratios of reduced quinone-to-pyocyanine concentrations, the decay became more rapid with increasing concentrations of reduced quinone. Estimation from the expanded time scale showed the decay constant of the rapid recovery to be 4, 2, and 0.8 msec, respectively, at the hydroquinone-to-pyocyanine ratios of 0.5, 1, and 2. The rate of the subsequent positive absorption change and its decay also increased with increasing concentrations of reduced quinone.

Concurrent with the rapid absorption decrease at 430 mμ, a rapid absorption decrease at 388 mμ was also observed (*vide infra*, see also Figure 10), the latter representing a rapid photoreduction of oxidized pyocyanine. The absorption changes at 432 and 388 mμ with continuous illumination shown in Figure 7 are consistent with the results obtained with flash illumination.

When the reaction mixture of Chl-PMS-UQ_6H_2-ethanol was made slightly alkaline by adding a trace amount of *p*H 7.5 Tris buffer, a reverse reaction occurred upon illumination. At 388 mμ, an absorption increase was observed instead of an absorption decrease (*vide infra*, see also Figure 10). At 430 mμ, only an absorption increase followed by a slow decay was observed. The initial rapid absorption decrease occurring in the acidic mixture was not observed in the alkaline system.

CHL–CYT *c* (OX.)–$TMQH_2$–ETHANOL SYSTEM. When PMS or pyocyanine in the systems described was replaced by oxidized cytochrome *c* (cytochrome *c* can be

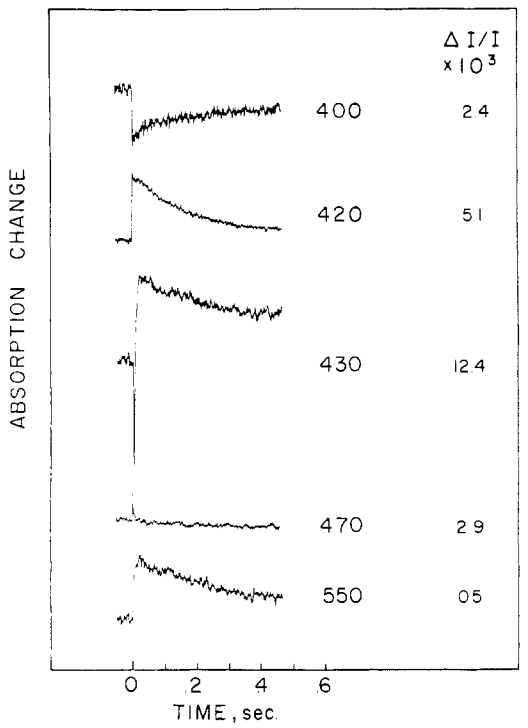

FIGURE 8: Absorption-change transients in the system Chl–Cyt *c* (ox.)–$TMQH_2$–ethanol at several selected measuring wavelengths. Concentrations used (in 3 ml solution): chlorophyll, 20 μg; oxidized cytochrome *c*, 0.05 μmole; $TMQH_2$, 0.3 μmole.

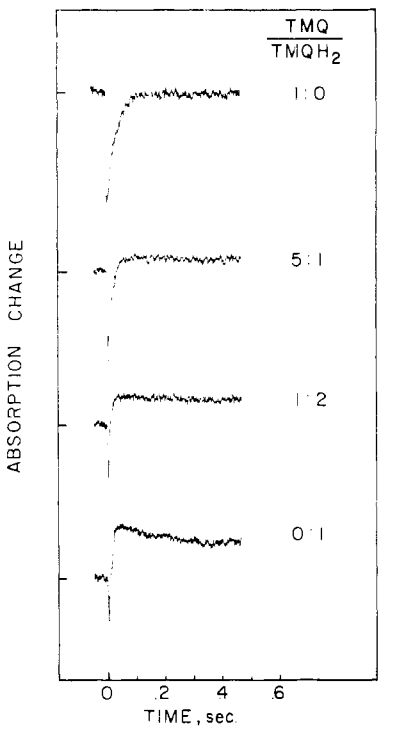

FIGURE 9: Effect of initial quinone composition on the absorption-change transients in the system Chl–Cyt *c* (ox.)–(TMQ, $TMQH_2$)–ethanol. Composition the same as in Figure 8 except the quinone is a mixture of oxidized and reduced compounds.

dissolved in ethanol containing as little as 1% water), a similar light-induced oxidation-reduction occurred. Upon flash illumination, rapid absorption changes occurred. Their wavelength profile again followed the triplet difference spectrum. Depending on the measuring-beam wavelengths, a slower absorption change followed by a slow decay also occurred. Typical absorption-change transients at a few selected wavelengths are shown in Figure 8. Interestingly, at 430 mμ the transient has practically the same complex profile as for the system Chl-Pyc-UQ_8H_2-ethanol. In the present case, the positive absorption increase with a slow decay at 430 mμ obviously was caused by cytochrome *c* reduction. This was confirmed by corresponding changes at 400, 420, and 550 mμ. At these wavelengths, where cytochrome *c* absorbs, only absorption changes followed by a slow decay were observed.

Varying $TMQH_2$ concentrations did not alter the kinetics of either the rapid or the slow reaction. However, the recovery time of the initial rapid absorption decrease or the rise time of the 550-mμ absorption increase was shortened from 1.05 to 0.5–0.6 msec when the cytochrome concentration was increased from 0.01 to 0.1 μmole/3 ml solution.

Another series of experiments was performed employing mixtures containing the same total quinone concentration but different proportions of TMQ and $TMQH_2$. The results are shown in Figure 9. With TMQ alone, the absorption decrease at 430 mμ was as rapid as usual, but the decay was much slower. Furthermore, the magnitude of the absorption decrease was the greatest with TMQ predominantly present in the system. There was negligible net positive absorption increase at 430 mμ. However, the 550-mμ transient showed that cytochrome *c* reduction occurred. With higher $TMQH_2$ concentrations in the quinone mixture, the decay of the initial absorption decrease and the decay of the positive absorption increase became more rapid and the magnitude of the positive absorption change also increased.

Discussion

Because of the intrinsically long lifetimes of the chlorophyll triplet, its possible participation in the primary energy-transfer processes in photosynthesis has long been suspected (Porter, 1963). Chlorophyll in the excited or metastable state could presumably undergo two types of charge-transfer reactions, either accepting or losing an electron, as exemplified by the Krasnovsky reaction (Krasnovsky, 1948) and the reaction with quinones or ferric salts (Rabinowitch and Weiss, 1937). Of these reactions, photooxidation in which excited chlorophyll functions as a reducing agent seems to be the most relevant to photosynthesis.

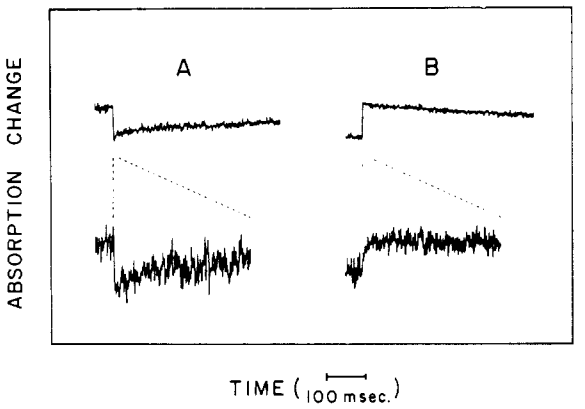

FIGURE 10: Absorption-change transients at 388 mμ for pyocyanine (left) in the system Chl–Pyc–UQ_6H_2–acidic ethanol and for PMS (right) in the system Chl–PMS–UQ_6H_2–alkaline ethanol (apparent *p*H, 7.15). Time scale applies to upper curves only. Lower curves are expanded-time-scale presentations for a small portion of the upper curves.

It should be noted that spectroscopic evidence alone does not allow one to determine whether excited chlorophyll forms a half-reduced or half-oxidized form, because both could lead to hindered resonance at the bridge carbons and result in reduced optical absorbance (Kamen, 1963). However, available evidence shows that chemical or photochemical oxidation can result in the disappearance of the absorption bands. For instance, chlorophyll *a* oxidation by ferric salts was studied some time ago by Rabinowitch and Weiss (1937). Linschitz and Rennert (1952) observed photobleaching of chlorophyll by quinones and reported that the bleached state survives in the dark at liquid nitrogen temperature. Goedheer (1960) showed that bacteriochlorophyll can be reversibly oxidized by ferric salts as well as a number of other oxidants. Chlorophyll present in a near-natural state as the far-red absorbing P700 also can undergo photochemical oxidation as well as chemical oxidation by ferricyanide (Kok, 1961). All these oxidation processes are accompanied by a disappearance of the original absorption bands.

In ethanolic systems containing chlorophyll alone or chlorophyll plus an oxidant, flash illumination causes bleaching of both the blue and red[2] absorption bands and an increase in the 470-mμ region. This similarity between the difference spectra of chlorophyll alone and chlorophyll plus an oxidant is not readily explainable. At first glance it would appear that the reaction observed with oxidant present is proceeding via the chlorophyll triplet, and the similar sensitivity to oxygen would agree with this. It is also conceivable, however, that in solution chlorophyll and the oxidant form an intimate complex, and the electronically excited chlorophyll rapidly transfers an electron to the oxidant molecule, forming a positive radical ion of chlorophyll and a semiquinone. In this case, triplet-state chlorophyll would not be formed. In any event, it is necessary to assume, from the experimental data, either that the difference spectra of the triplet state and the positive radical ion of chlorophyll are almost identical, or that what has been considered to be the absorption spectrum of triplet-state chlorophyll is actually that of a chlorophyll positive radical ion formed by reaction with solvent molecules. Without specifying the early photochemical sequence any further, it is possible to write the following reaction scheme:

$$\text{Chl} \xrightarrow{h\nu} \text{Chl}^*$$

$$\text{Chl}^* + \text{Q} \longrightarrow \text{Chl}^+ + \text{Q}^- \longrightarrow \text{Chl} + \text{Q}$$

In the dark, the positive ion of chlorophyll and the semiquinone back-react to restore the original state.

Similar systems consisting of chlorophyll and various quinones, including UQ_6, have been examined with the EPR technique by Tollin and Green (1962, 1963), who reported signals characteristic of semiquinone free radicals. Action spectra of these reactions show that radical production results from light energy absorbed by chlorophyll. Furthermore, the reaction is completely reversible. The EPR signals were interpreted as due to semiquinones formed by an electron transfer from the excited chlorophyll to the quinones.

In the systems consisting of chlorophyll plus a redox couple, the difference spectra for the rapid initial absorption changes also follow the chlorophyll triplet pattern. Furthermore, a concurrent rapid absorption decrease at 388 mμ, representing the reduction of pyocyanine or PMS, can also be observed. This is shown on the expanded time scale in Figure 10, left. The rapidity with which these initial absorption changes take place is limited only by the time resolution of our measuring instrument, namely, $\leq 10^{-4}$ second. Thus for the system Chl-Pyc-UQ_6H_2-ethanol (Figure 4), one may interpret the initial rapid absorption decrease at 430 mμ as consisting of chlorophyll excitation followed by an instantaneous transfer of an electron from chlorophyll to pyocyanine to form pyocyanine semiquinone. The rapid return would then represent the reaction between the positive radical ion of chlorophyll and reduced quinone to regenerate chlorophyll plus a semiquinone of UQ_6. This is also consistent with the results shown in Figure 6, that the return rate is accelerated by increasing concentrations of UQ_6H_2. The net positive change in absorption at 430 mμ would then be a measure of the amount of the stable semiquinonoid pyocyanine formed. The slow decay probably represents the recombination of the semiquinones. The reaction sequence may be represented by:

$$\text{Chl} \xrightarrow{h\nu} \text{Chl}^*$$

[2] Simultaneous bleaching of both the blue and red absorption bands and an identical kinetics in their recovery have been observed in a separate set of experiments with a conventional flash-photolysis apparatus.

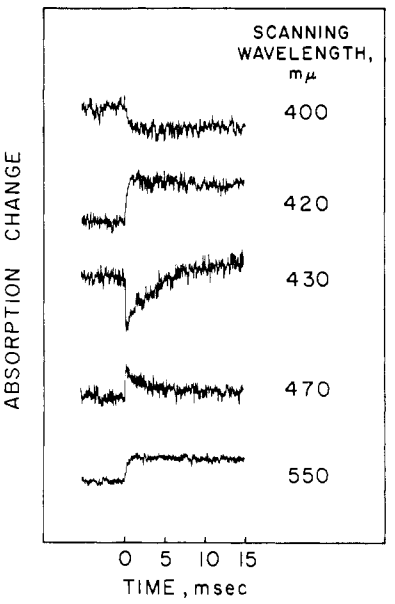

FIGURE 11: Same as Figure 8 except presented on an expanded time scale, as indicated.

$$Chl^* + Pyc \longrightarrow Chl^+ + Pyc^-$$

$$Chl^+ + QH_2 \longrightarrow Chl + QH\cdot + H^+$$

$$QH\cdot + H^+ + Pyc^- \longrightarrow QH_2 + Pyc$$

Recent independent findings from a study of chlorophyll-sensitized dye reduction (Seely, 1965) are in agreement with the foregoing reaction sequence. It has been shown that, in the chlorophyll-sensitized photoreduction of methyl red by ascorbate, the initial photoreaction involves the transfer of an electron from the excited chlorophyll to the dye. This conclusion was drawn from the fact that photoreduction of chlorophyll by ascorbate alone proceeds at a much slower rate than that of dye reduction.

When the Chl-PMS-UQ_6H_2-ethanol system is made slightly alkaline with Tris buffer, the reverse reaction occurs. This is indicated by an absorption increase at 388 mμ (Figure 10, right), representing PMS oxidation. Furthermore, as shown on the expanded time scale in Figure 10, right bottom, the rise time of this absorption change is much longer than 10^{-4} second, indicating that PMS was not involved in the initial electron-transfer step. As mentioned earlier, no rapid absorption decrease was observed in the alkaline ethanolic mixture; the reason for its absence is not yet obvious.

In the Chl–Cyt *c* (ox.)–$TMQH_2$–ethanol system, the difference spectra plotted from individual absorption-change transients, such as those shown in Figure 8, conclusively show that cytochrome *c* was reduced. Cytochrome *c* reduction is represented by the slowly decaying transients, while the rapidly decaying transients follow the chlorophyll triplet pattern. Time expansion of these individual transients, as shown in Figure 11, indicates that cytochrome reduction occurred in the dark, since the rise-times of the cytochrome transients are of the order of 10^{-3} seconds. If this is the case, the available evidence would allow one to postulate that the initial electron transfer could be either a loss of an electron to the oxidized quinone (some would be present in the oxidized form) or a gain of an electron from the reduced quinone by the excited chlorophyll. However, parallel reactions in the Chl-Pyc-UQ_6H_2-ethanol system, and in others, favor the case for formation of a positive radical ion of chlorophyll in the initial electron-transfer step. Thus the reaction sequence involving cytochrome *c* may be represented by the following scheme:

$$Chl \xrightarrow{h\nu} Chl^*$$

$$Chl^* + TMQ \longrightarrow Chl^+ + TMQH\cdot$$

$$Chl^+ + TMQH_2 \longrightarrow Chl + TMQH\cdot$$

$$TMQH\cdot + \text{Cyt } c \text{ (ox.)} \longrightarrow TMQ + \text{Cyt } c \text{ (red.)}$$

The over-all reaction scheme is consistent with the difference spectra of the slowly decaying and rapidly decaying transients. Results shown in Figures 8 and 11 support the case of cytochrome reduction in the dark. Results shown in Figure 9 further support the suggestion that TMQ participates in the initial electron-transfer reaction and that $TMQH_2$ participates in the back-reaction.

It is obvious that further experimentation is needed to ascertain completely the proposed reaction mechanism. One area of special interest would be to examine the reaction course of the quinones by following the absorption-change transients in the ultraviolet region where the quinones absorb. Work along this line is in progress.

Acknowledgment

The authors thank Miss Elena Ngo for technical assistance in these experiments. Thanks are also due to Dr. W. S. Zaugg for furnishing reduced ubiquinone and to Dr. H. E. Davenport for furnishing pyocyanine.

References

Claesson, S., Lindquist, L., and Halström, B. (1959), *Nature 183*, 661.

Goedheer, J. C. (1960), *Biochim. Biophys. Acta 38*, 389.

Green, D. E., and Burkhard, R. K. (1961), *Arch. Biochem. Biophys. 92*, 312.

Kamen, M. D. (1963), Primary Processes in Photosynthesis, New York, Academic, p. 156.

Ke, B., Treharne, R. W., and McKibben, C. (1964), *Rev. Sci. Instr. 35*, 296.

Kok, B. (1961), *Biochim. Biophys. Acta 48*, 527.

Krasnovsky, A. A. (1948), *Dokl. Akad. Nauk SSSR 60*, 421.

Krasnovsky, A. A. (1955), *Dokl. Akad. Nauk SSSR 103*, 283.
Krasnovsky, A. A. (1961), *Ann. Rev. Plant Physiol. 12*, 363.
Linschitz, H., and Rennert, J. (1952), *Nature 169*, 193.
Linschitz, H., and Sarkanen, K. (1958), *J. Am. Chem. Soc. 80*, 4826.
Livingston, R. (1955), *J. Am. Chem. Soc. 77*, 2179.
Livingston, R. (1960), *Quart. Rev. (London) 14*, 174.
Livingston, R., and Ryan, V. A. (1953), *J. Am. Chem. Soc. 75*, 2176.
Nieman, R. H., and Vennesland, B. (1959), *Plant Physiol. 34*, 255.
Porter, G. (1963), *Proc. Roy. Soc. (London), Ser. B: 157*, 293.
Rabinowitch, E., and Weiss, J. (1937), *Proc. Roy. Soc. (London), Ser. A: 162*, 251.
Seely, G. R. (1965), *J. Phys. Chem. 69* (in press).
Tollin, G., and Green, G. (1962), *Biochim. Biophys. Acta 60*, 524.
Tollin, G., and Green, G. (1963), *Biochim. Biophys. Acta 66*, 308.
Vernon, L. P. (1961a), *Acta Chem. Scand. 15*, 1645.
Vernon, L. P. (1961b), *Acta Chem. Scand. 15*, 1651.
Vernon, L. P. (1963), *in* Bacterial Photosynthesis, Gest, H., San Pietro, A., and Vernon, L. P., eds., Yellow Springs, Ohio, Antioch Press, p. 235.
Vernon, L. P., Shaw, E., Zaugg, W. S., and Ke, B. (1964), *Federation Proc. 23*, 227.
Vernon, L. P., Zaugg, W. S., and Shaw, E. (1963), *Natl. Acad. Sci.–Natl. Res. Council, Publ. 1145*, 509.
Zaugg, W. S. (1963), *Proc. Natl. Acad. Sci. U.S. 50*, 100.
Zaugg, W. S. (1964), *J. Biol. Chem. 239*, 3964.
Zaugg, W. S., Vernon, L. P., and Tirpak, A. (1964), *Proc. Natl. Acad. Sci. U.S. 51*, 232.
Zieger, G., and Witt, H. T. (1961), *Z. Physik. Chem. (Frankfurt) 28*, 273.
Zscheile, F. P., and Comar, C. L. (1941), *Botan. Gaz. 102*, 463.

Peroxidase-catalyzed Oxidation of Indole-3-acetic Acid*

R. L. Hinman and J. Lang

ABSTRACT: A thorough study of the products of the *in vitro* oxidation of indole-3-acetic acid (IAA), catalyzed by horseradish peroxidase in the absence of added hydrogen peroxide, has shown that at substrate concentrations of 2×10^{-4} M and below 3-methyleneoxindole is the end product and oxindole-3-carbinol is its precursor. To explain these products a reaction sequence for peroxidase-catalyzed oxidation of IAA is proposed in which the peroxidase functions as a one-electron oxidizing agent and an indolenine hydroperoxide is the first intermediate. The hydroperoxide is converted to oxindole-3-carbinol via an indolenine epoxide.

The oxidation of IAA is concentration dependent and at higher concentrations a neutral indole appears to be the principal product. Oxidation of indole-3-alkanoic acids by a variety of oxidizing agents shows that 3-alkylideneoxindoles are very common products of oxidation of 3-alkylindoles, but that several pathways are possible for the transformation.

The existence of enzyme systems in higher plants that catalyze the oxidative degradation of the plant-growth hormone indole-3-acetic acid (IAA)[1] has been recognized for many years, but the detailed pathway of auxin destruction has not been elucidated. The general status of the problem has been reviewed by Ray (1958).

On the other hand considerable progress has been made toward understanding the enzymes involved. These appear to be peroxidases (Ray, 1958, 1960) and this peroxidase-catalyzed oxidation of IAA is of particular interest as one of the few known cases in which oxygen is consumed and exogenous hydrogen peroxide is not required (Mason, 1957). In addition, it has recently been suggested that the oxidation may play a role in the growth-regulating functions of IAA (Kefford *et al.*, 1963).

In an effort to elucidate the reaction path, we have studied the behavior of IAA and a variety of related indolic materials in the presence of peroxidase from horseradish. A number of chemical models of the peroxidase system have also been designed to furnish additional information. In general, reactions have been followed and ultimate products have been identified where possible by ultraviolet absorption measurements. This method permits examination of unstable intermediates and products in the reaction mixture and minimizes the possibility of further reactions which may accompany isolation techniques (Ray, 1958).

* From the Union Carbide Research Institute, Union Carbide Corporation, Tarrytown, N.Y. *Received August 24, 1964.*

[1] Abbreviation used in this work: IAA, indole-3-acetic acid.