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LIGHT-INDUCED SINGLE ELECTRON-TRANSFER REACTIONS BETWEEN CHLOROPHYLL *a* AND QUINONES IN SOLUTION

II. SOME EFFECTS OF NON-QUINONOID DONORS AND ACCEPTORS: RIBOFLAVIN, THIOCTIC ACID AND NADH

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SUMMARY

1. Light-induced single electron-transfer reactions have been observed in two-component systems involving chlorophyll and riboflavin or thioctic acid and in three-component systems involving NADH, chlorophyll, and benzoquinone or riboflavin.

2. In the chlorophyll–riboflavin system, illumination of acidic solutions leads to the formation of the semiquinone of riboflavin and, via a side reaction, to the destruction of chlorophyll.

3. In the chlorophyll–thioctic acid system, excitation of thioctic acid produces a species (perhaps the biradical resulting from sulfur–sulfur bond cleavage) capable of forming free radicals upon interaction with an electronically excited chlorophyll molecule.

4. In the three-component systems, from 2- to 8-fold enhancements in steady-state radical concentrations can be achieved by adding NADH to solutions of chlorophyll and acceptors.

INTRODUCTION

In a previous paper[1] we have examined some of the characteristics of unpaired electron formation in illuminated chlorophyll–quinone solutions. Radical production in these systems has been attributed to the occurrence of single electron-transfer reactions between electronically excited chlorophyll molecules and quinone molecules, and a fairly detailed mechanism has been proposed to account for the results. A number of questions have arisen out of this work. The present study has been directed to two of these: (a) can substances other than quinones function as electron acceptors from chlorophyll?; (b) what effect will the presence of a third component, either donor or acceptor, have upon the electron transfer between chlorophyll and quinone?

Abbreviation: EPR, electron paramagnetic resonance.

MATERIALS AND METHODS

EPR spectra have been obtained as indicated previously[1]. Optical absorption spectra were recorded using a Cary model 11 spectrophotometer.

Except where otherwise stated, illumination of the samples was carried out using light of wavelengths longer than 5500 Å in order to avoid absorption by [illegible] other than chlorophyll.

All other experimental techniques were as outlined in the previous work[1].

Chlorophylls *a* and *b* (puriss.) (in all of the systems we have examined, chlorophyll *b* functions in essentially the same manner as does chlorophyll *a*) were obtained from Fluka A.G.; riboflavin and thioctic acid were obtained from California Corporation for Biochemical Research; NADH was obtained from Sigma Chemical Co. All compounds were used without further purification. Spectrophotometric assay indicated that the NADH was approx. 75 % pure.

RESULTS AND DISCUSSION

(1) The chlorophyll–riboflavin system

When carefully degassed solutions of riboflavin* and chlorophyll (*a* or *b*) in absolute ethanol (saturated with respect to riboflavin; 10^{-4} M in chlorophyll) were illuminated for several minutes in the EPR spectrometer, only very small, poorly resolved, signals were observed. It is known that the protonated form of riboflavin is a much better oxidizing agent than is the neutral species[2,3] and that the riboflavin semiquinone is considerably more stable in acidic than in neutral solution[3]. Furthermore, riboflavin is quite a bit more soluble in acid solvents than in absolute ethanol. With these considerations in mind, we performed similar experiments to the above using acidified ethanol (prepared by saturating absolute ethanol with gaseous HCl), containing higher concentrations of riboflavin (10^{-3} M), as the solvent. With these systems, large EPR signals were observed.

No signals were obtained with riboflavin alone under these conditions, nor were signals present in the dark prior to illumination, demonstrating that it is illumination of chlorophyll which is effective in producing the radicals. However, when riboflavin solutions were illuminated with blue light (absorbed by riboflavin), very large signals were produced which were identical to those obtained when chlorophyll was present and red light was used.

In Fig. 1 we compare an EPR spectrum obtained from an illuminated chlorophyll–riboflavin solution at room temperature with a spectrum obtained upon Zn reduction of riboflavin in acidic solution. It is apparent that the two spectra are essentially identical. Hence, we may identify the free radical formed upon illumination, both in the presence and in the absence of chlorophyll, as the semiquinone of riboflavin.

The steady-state radical concentration obtained upon illumination was found to increase with decreasing temperature, down to about −30°, although below about −10° no resolution of hyperfine structure was apparent. A more complete temperature-dependence study was not carried out.

* Several other flavin analogs behave similarly to riboflavin, *e.g.*, 9-methyl isoalloxazine, alloxazine, and 6,7-dimethyl alloxazine (lumichrome).

In both the presence and absence of chlorophyll, the radical signal did not decay measurably, over a period of many hours, when the light was turned off. This is in marked contrast to the chlorophyll–quinone systems[1] which exhibit rapidly decaying signals in the dark, except at very low temperatures.

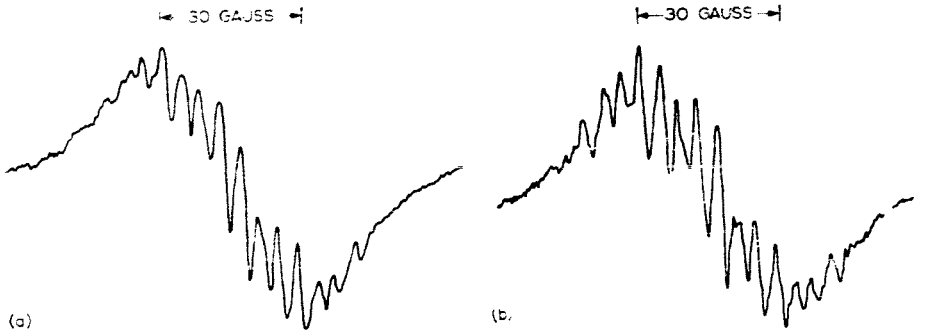


Fig. 1. a, EPR spectrum of an illuminated degassed solution of chlorophyll *a* ($1 \cdot 10^{-4}$ M) and riboflavin ($1 \cdot 10^{-3}$ M) in ethanol–HCl at room temperature. The same spectrum is obtained upon illumination of solutions of riboflavin alone with blue light. b, EPR spectrum of a solution of riboflavin in ethanol–HCl reduced with Zn at room temperature.

The illuminated flavin solutions appeared orange or orange-brown in color, in contrast to the unilluminated solutions which were either yellow or bright green in color, depending upon whether chlorophyll was absent or present. When the illuminated solutions were opened to the air, the original green or yellow color gradually returned (over a period of several hours) and the paramagnetism disappeared. The same result could be accomplished by bubbling air or pure O_2 through the solution. If these aerated solutions were then degassed again, a similar pattern could be obtained upon reillumination.

In order to obtain further insight into the nature of the processes occurring in these solutions, optical absorption spectroscopy was utilized. In Fig. 2 is shown a series of such spectra obtained upon illumination of chlorophyll–riboflavin and chlorophyll solutions at room temperature. In the flavin-containing system, we have observed a decrease in the chlorophyll absorption at 665 mμ and the formation of new absorption bands at approx. 485 mμ and 575 mμ. With chlorophyll alone, we obtained a much more rapid decrease in the 665-mμ band and an increase in absorbancy at approx. 515 mμ and 575 mμ.

In Fig. 3 is shown the absorption spectrum, in the long-wavelength region, of an illuminated (with white light) riboflavin solution. A maximum at 485 mμ, which is completely absent in the unilluminated solution*, is apparent. It is this band which produces the above-mentioned color changes.

From the above data we conclude that the new shoulder which appeared in the absorption spectrum of illuminated chlorophyll–riboflavin solutions must consist of components due partly to flavin and partly to chlorophyll transformation products, although the flavin probably contributes the largest fraction.

* In strongly acidic solution, riboflavin exhibits a single absorption maximum in the visible spectral region at 390 mμ.

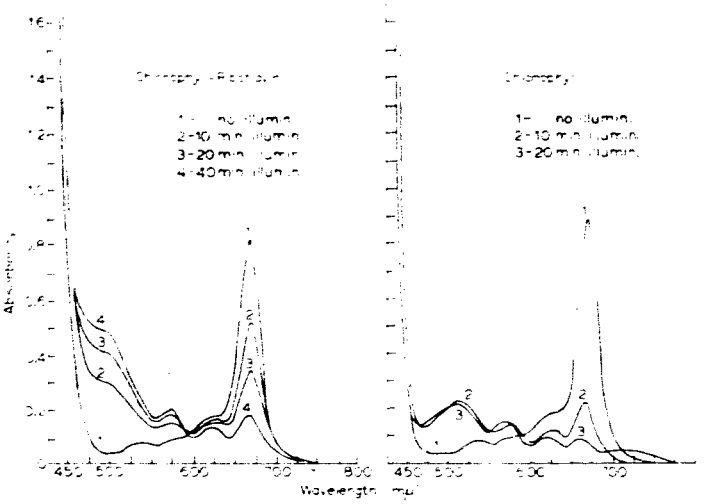


Fig. 2. Optical absorption spectra of degassed solutions of chlorophyll *a* and riboflavin and chlorophyll *a* alone in ethanol–HCl as a function of time of illumination with red light at room temperature. Curve 1, no illumination; curve 2, 10-min illumination; curve 3, 20-min illumination; curve 4, 40-min illumination.

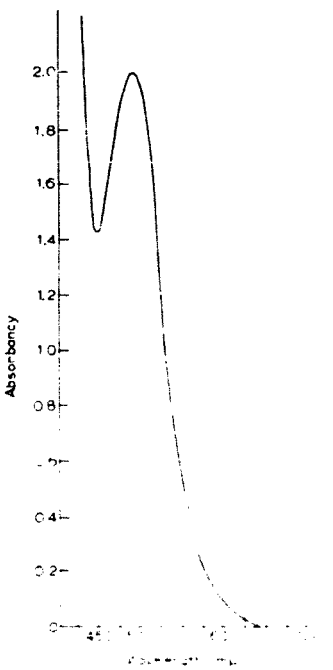


Fig. 3. Optical absorption spectrum of riboflavin in ethanol–HCl (degassed) after a 10-min illumination period with white light.

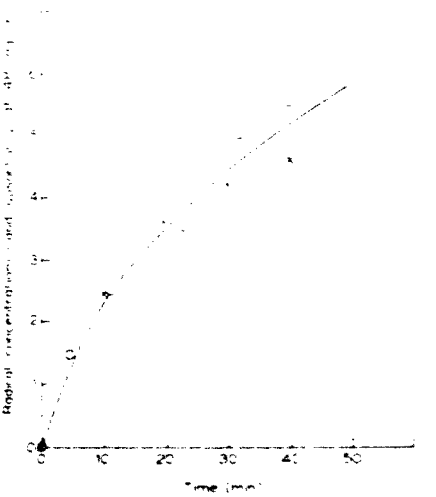


Fig. 4. Growth curves for paramagnetism and absorbancy at 485 mμ in illuminated degassed chlorophyll–riboflavin solutions in ethanol–HCl.

In Fig. 4 we have compared the growth curves for the paramagnetism* and for the shoulder at 485 mμ in illuminated chlorophyll–riboflavin solutions. The fact that these are approximately the same indicates that the two effects are the result of the formation of the same species, namely the riboflavin semiquinone. This interpretation is consistent with the work of BEINERT[3,4] on the optical spectra of flavin semiquinones in acid solution.

That the absorbancy at 665 mμ decreases in both the presence and the absence

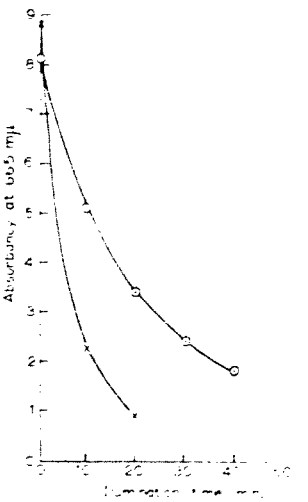


Fig. 5. Effect of illumination with red light on chlorophyll *a* absorption at 665 mμ in ethanol–HCl (degassed) at room temperature. ⊙—⊙, chlorophyll–riboflavin; ×—×, chlorophyll.

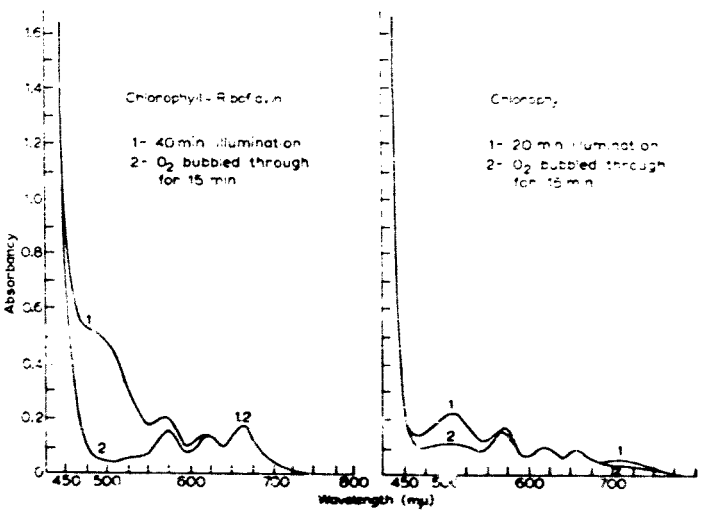


Fig. 6. Optical absorption spectra of illuminated (red light) chlorophyll *a* and chlorophyll–riboflavin solutions before and after bubbling with O_2.

* The extreme slowness of the rise in paramagnetism is again in marked contrast to the chlorophyll–quinone systems which exhibit quite rapid rise times (of the order of seconds), except at very low temperatures[1].

of riboflavin indicates that this process is independent of riboflavin reduction. This interpretation is further substantiated by the data in Fig. 5 in which we have plotted the decrease in absorbancy at 665 mμ as a function of illumination time in both systems It is apparent that the presence of riboflavin markedly decreased the rate of disappearance of the chlorophyll, indicating that chlorophyll destruction and riboflavin reduction by excited chlorophyll molecules are competitive processes.

In Fig. 6 we have plotted the optical absorption spectra of illuminated chlorophyll–riboflavin and chlorophyll solutions before and after bubbling with pure O_2. After the O_2 treatment, the 485-mμ band in the chlorophyll–riboflavin solution completely disappeared. This is consistent with our interpretation of the origin of this band in terms of the riboflavin semiquinone. The 515-mμ band in the chlorophyll solution also disappeared to some extent, indicating that it too is due to a reduced species. On the other hand, the main chlorophyll peak at 665 mμ was not at all regenerated. Thus, chlorophyll itself has been irreversibly destroyed. Similar results as the above were obtained upon allowing the illuminated solutions to stand open to the air for long periods (several hours).

(2) The chlorophyll–thioctic acid system

BARLTROP, HAYES AND CALVIN[5] have obtained evidence for the photolytic cleavage of sulfur–sulfur bonds in ethanol solutions of thioctic acid and related compounds. Furthermore, it was suggested, on theoretical grounds, that chlorophyll could sensitize such cleavage reactions. These results, in conjunction with the fact that a role for thioctic acid in the primary quantum conversion process of photosynthesis has been postulated[6], have led us to examine illuminated solutions of chlorophyll and thioctic acid in the EPR spectrometer.

Degassed solutions of thioctic acid alone in ether–isopentane–alcohol were found to give a small broad signal in the dark at low temperatures which did not change upon illumination with white light. In the presence of chlorophyll, however, although essentially the same small signal was observed in the dark, there was a significant increase in paramagnetism upon illumination with red light. This is shown in Fig. 7.

When the chlorophyll–thioctic acid samples were illuminated with white light, the signal grew to several times its original height. This experiment is also shown in Fig. 7.

The fact that a single-line EPR spectrum was observed is consistent with the unpaired electron residing on a sulfur atom, although of course it does not prove this.

When the light was turned off, the signal rapidly decayed to the original dark value. Rise and decay curves are shown in Fig. 8. It is apparent that there is an initial very fast rise followed by a much slower rise, with an identical pattern being observed in the decay.

If the chlorophyll–thioctic acid sample, which had previously been illuminated with white light, was illuminated with red light, a considerably larger steady-state signal was observed than was obtained using a sample which had never been exposed to white light. The comparison is shown in Fig. 9. Furthermore, if the sample, which had been illuminated with white light in the cold (−100°), was warmed to room temperature, recooled, and then illuminated with red light, only a small signal was observed, comparable to that obtained with a previously unilluminated sample. Thus, white light produced a species, not produced by red light, which was stable for at

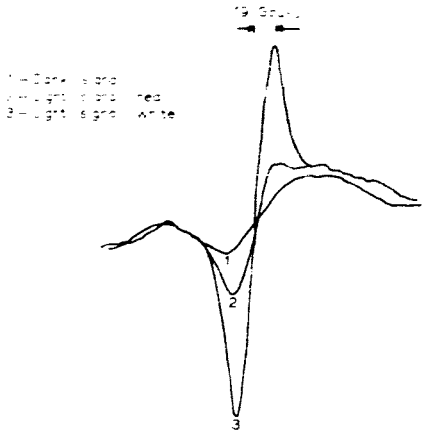


Fig. 7. Effect of illumination upon EPR signals in chlorophyll *a* ($1 \cdot 10^{-4}$ M)–thioctic acid (satd.) solutions (degassed) in ether–isopentane–alcohol at —100°.

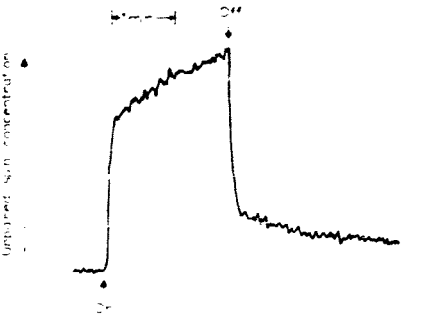


Fig. 8. Rise and decay curves for paramagnetism in illuminated chlorophyll–thioctic acid solutions in ether–isopentane–alcohol at —100°.

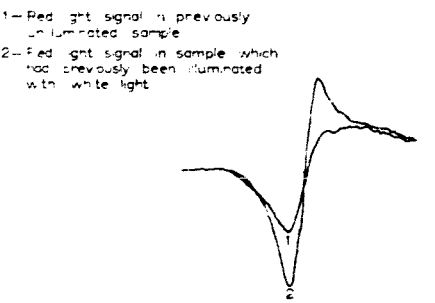


Fig. 9. Comparison between EPR signal produced in chlorophyll–thioctic acid solutions in ether–isopentane–alcohol at—100° by illumination with red light with and without prior white-light illumination.

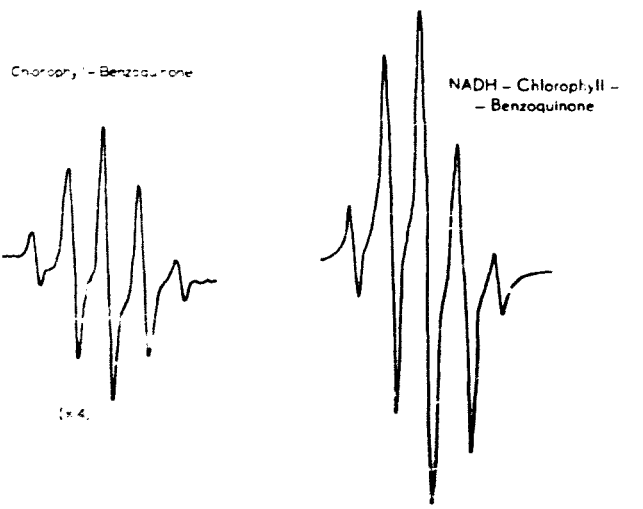


Fig. 10. Effect of presence of NADH ($4 \cdot 10^{-4}$ M) upon steady-state light-induced EPR signal in chlorophyll *a* ($4 \cdot 10^{-4}$ M)–benzoquinone ($4 \cdot 10^{-2}$ M) solutions (degassed) in ethanol–water (11:1) at —50°.

least several minutes in the dark at low temperatures and which, although diamagnetic itself (or at least unobservable in EPR under our conditions), can form free radicals upon interaction with an excited chlorophyll molecule. Further, this species decays in the dark at room temperature. This sequence of events could be repeated upon reillumination with white light.

Experiments using various Corning glass filters in the light path have shown that it is light between 3200 Å and 4000 Å which is effective in producing the above mentioned species. Furthermore, illumination with these wavelengths does not in itself produce a radical signal. It is significant that the optical absorption spectrum of thioctic acid in ether–isopentane–alcohol shows a broad band between 3000 Å and 4000 Å (λ_{max} about 3300 Å). Thus, we may tentatively conclude that excitation of the thioctic acid component produces a species which forms radicals only upon interaction with an electronically excited chlorophyll molecule (see below for further discussion).

(3) Three-component systems: donor–chlorophyll–acceptor

Part of our interest in systems of this type comes from recent theories of the primary process of photosynthesis[7,8] in which light-induced electron-transfer processes from donor to chlorophyll to acceptor are postulated. If such reactions could occur *in vitro*, one might expect that the effect of the presence of an electron donor in an illuminated chlorophyll–acceptor solution would be observable by EPR techniques.

In Fig. 10 is shown the effect of the presence of NADH on the steady-state radical concentration of an illuminated degassed chlorophyll–benzoquinone solution in aqueous ethanol. Approximately an 8-fold enhancement in signal height over the control was obtained. Experiments using a Bausch and Lomb grating monochromator in the light path have shown that it was light absorbed by chlorophyll which produced the radicals.

It is of interest that the only EPR signal observed in these systems was that due to the one-electron reduction product of the quinone moiety. No signal was observed for any radical species derived from either chlorophyll or NADH. This is similar to the failure to observe chlorophyll radicals in the two-component systems[1], and is probably due to the same reasons.

When solutions containing only NADH and chlorophyll were illuminated, a very small broad signal was obtained. The identity of the species giving rise to this signal is uncertain.

We have observed that the addition of NAD^+ to solutions of chlorophyll and benzoquinone has essentially no effect on the production of unpaired spins by light.

It is worth noting that a small benzosemiquinone signal can be observed in a NADH–benzoquinone solution in the dark in the absence of chlorophyll. This is probably due to a redox reaction occurring spontaneously between these two species.

When NADH was added to degassed solutions of chlorophyll and riboflavin in acidic ethanol, a 2-fold enhancement in steady-state radical concentration was obtained. Again, the radical obtained was exclusively that characteristic of the acceptor species, *i.e.*, the riboflavin semiquinone. Also, as in the previous system, a small semiquinone signal was observed in an NADH–riboflavin solution in the dark, indicating a spontaneous reaction between the two species. Furthermore, a small

effect of illumination with red light was noted in this case, the final steady-state signal being approx. one-sixth that obtained with the three-component system. Inasmuch as neither NADH nor riboflavin absorb in this wavelength region, the possible occurrence of complexes is suggested.

In addition to NADH, we have tried using hydroquinone and tetrachlorohydroquinone as electron donors in ether–isopentane–alcohol solutions of chlorophyll and quinones. In both of these cases, the donors apparently reacted preferentially with the acceptors in the dark.

It is of interest to note that the EPR signal observed with 1:1 mixtures of tetrachlorohydroquinone and benzoquinone in ether–isopentane–alcohol in the dark was that due solely to the tetrachlorobenzosemiquinone species. No signal was observed for the benzosemiquinone species. We have also observed a similar result when 9,10-phenanthrenequinone was used as acceptor, *i.e.*, in a mixture of hydroquinone and this compound, only the benzosemiquinone radical is observed. Under no conditions have we been able to observe both possible semiquinone species simultaneously. The possible reasons for this remain obscure.

We have also carried out a few experiments in which more than one quinone was present simultaneously with chlorophyll. In every case, only one semiquinone, corresponding to the stronger oxidizing agent of the pair, was formed.

CONCLUSIONS

(1) The chlorophyll–riboflavin system

We have shown above that illumination of a degassed solution of chlorophyll and riboflavin in ethanol–HCl leads to the formation of the semiquinone of riboflavin and, via a side reaction, to the destruction of chlorophyll. Inasmuch as chlorophyll decomposition (as measured by the absorbancy change at 665 mμ) is considerably slower in the presence of riboflavin than in its absence, we must conclude that the chlorophyll is either completely or partially conserved in the process of riboflavin semiquinone formation. The following scheme is consistent with this fact and with the mechanism suggested in the earlier work with quinones[1]:

$$C + h\nu \leftrightharpoons C^* \qquad (1)$$

$$C^* + R \longrightarrow C^+ + R^- \qquad (2)$$

or

$$C^* \xrightarrow{\text{solvent}} \text{products} \qquad (2a)$$

$$C^+ \xrightarrow{\text{solvent}} C \qquad (3)$$

$$2C^+ \xrightarrow{\text{solvent}} C + X \qquad (3a)$$

$$R + h\nu \xrightarrow{\text{solvent}} R^- \qquad (4)$$

C = chlorophyll; R = riboflavin; C^+ = chlorophyll positive ion-radical; R^- = riboflavin semiquinone. An asterisk denotes an electronically excited state.

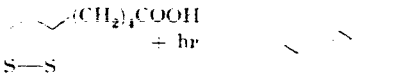
According to this formulation, an electronically excited chlorophyll molecule (formed by Reaction 1) can react either with riboflavin to form the semiquinones (Reaction 2) or with solvent to form unknown products (Reaction 2a). The chlorophyll positive ion formed in Reaction 2 can then regenerate chlorophyll completely (as in

Reaction 3) or partially (as in Reaction 3a). Further work will be necessary in order to substantiate this mechanism.

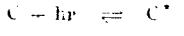
It is of interest to note that riboflavin itself can be reduced upon illumination with blue light. Presumably, the solvent plays a role here (Reaction 4).

(2) The chlorophyll–thioctic acid system

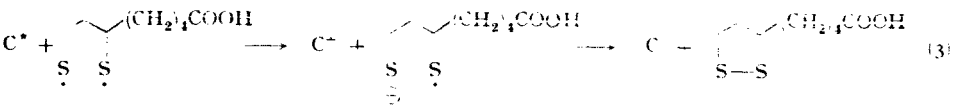
We have shown above that excitation of thioctic acid produces a species capable of forming free radicals upon interaction with an electronically excited chlorophyll molecule. This can be made consistent with the work of BARLTROP, HAYES AND CALVIN[5] and with our earlier work[1] as follows:



(1)



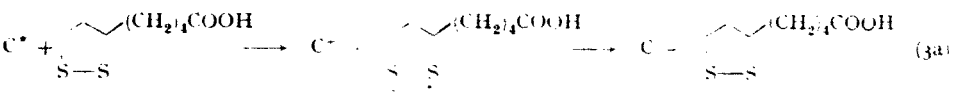
(2)



(3)

We must assume here that the biradical formed in Reaction 1 is undetectable in our present system and that it is stable enough at low temperatures to survive for a matter of minutes. Of course, we cannot at this stage rule out the possibility that the electron acceptor from chlorophyll is not the biradical but some compound derived from the reaction of this species and the solvent.

The fact that excitation of chlorophyll alone is capable or producing a smaller, but still detectable, quantity of the radical produced in Reaction 3 above suggests that sulfur–sulfur bond cleavage and electron transfer can occur in one step, albeit with reduced efficiency, as follows:



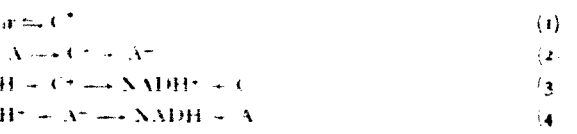
(3a)

If this is indeed so, it would be consistent with the postulated participation of thioctic acid in the primary quantum-conversion process of photosynthesis[6]. More work is needed to establish this.

It should be pointed out that the nature of the species giving rise to the dark signal found in the thioctic acid solutions is completely obscure at present.

(3) Three-component systems

The experiments reported here have demonstrated that one can induce 2–8-fold enhancements in steady-state radical concentrations by adding NADH to solutions of chlorophyll and acceptors. The simplest interpretation of these effects is in terms of the following scheme:



The essential feature of the enhancement thus lies in the regeneration of chlorophyll from C^+ via Reaction 3. This allows chlorophyll to react with more acceptor (it is to be recalled that the acceptor is present in excess in these systems). The overall result of these processes is the transfer of an electron from donor (NADH) to acceptor (A) via a chlorophyll-photosensitized oxidation–reduction. This type of reaction, although occurring in an ordered matrix, is precisely what is postulated in the most recent theories of the primary process of photosynthesis[7,8].

(4) Some general comments

It is apparent from this and from the previous work[1] that many of the components known to be present in chloroplasts and to participate in photosynthesis can react with electronically excited chlorophyll molecules to produce free radicals. Inasmuch as light-induced radical formation appears to be a characteristic property of photosynthetic materials[9–12], we have felt it to be of importance to explore the possible interactions which can occur *in vitro*. It remains to be shown that these reactions are efficient enough to be of significance in terms of photosynthesis. We are at present engaged in this aspect of the problem. It is, of course, obvious that this type of work can at best suggest what might be occurring within the chloroplast. The final arbiter will be studies *in vivo*.

ACKNOWLEDGEMENT

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