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CHEMICAL TRAPPING OF A PRIMARY QUANTUM CONVERSION PRODUCT IN PHOTOSYNTHESIS*

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The capacity of photosynthetic organisms to exhibit photo-induced electron paramagnetic resonance (EPR) signals has been known for over ten years.[1] Subcellular units of photosynthetic materials, the quantasomes and the chromatophores, are capable of Hill Reaction activity, and also of exhibiting the light-induced EPR signals. This, coupled with the rapid rise and decay kinetics of these signals,[2] suggests but does not prove that the unpaired electrons are involved in the initial electron transfer processes in the primary quantum conversion act.

The identification of the species giving rise to these signals and their connection with processes of primary quantum conversion remains elusive even though such varied approaches as mutant strains,[3] special growth conditions,[4] extreme physical conditions,[5] special metabolic inhibitors,[6] etc. have been applied to this problem. In this communication we wish to report another method being used in an attempt to identify the species responsible for the unpaired electrons.

Hoffman prepared a water soluble, stable free radical, di-tertiary-butylnitroxide (hereafter called DTBN), which is a "vigorous free radical scavenger".[7] It shows a sharp, well resolved, symmetrical, three-line paramagnetic resonance spectrum that is relatively insensitive to the molecular environment. The chemistry of di-tertiarybutylnitroxide has not been studied

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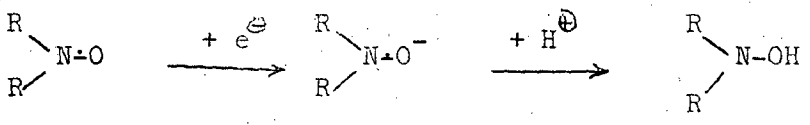
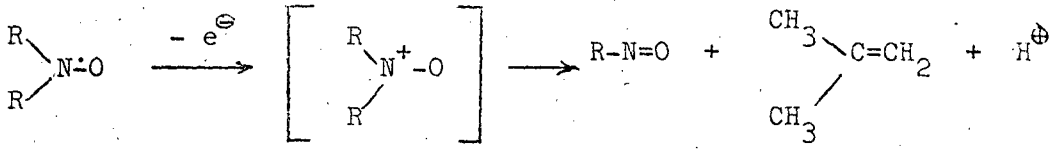
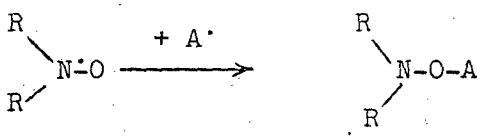
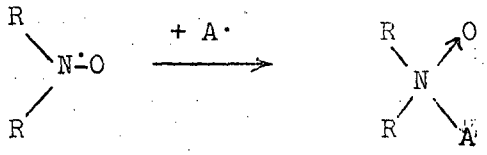
extensively. However, four distinct types of interaction can be envisioned for this molecule. It could undergo a one-electron reduction to form a hydroxylamine which can be reduced subsequently to the amine; an oxidative degradation to 2-methyl-2-nitrosopropane and isobutylene; or a coupling with another radical forming either an oxygen substituted hydroxylamine or a trisubstituted amine oxide. The reactions are represented by equations I through IV.

I) 

II) 

III) 

IV) 

R = tertiary butyl group

A· = unspecified radical

The present work sought to determine whether, in the presence of photosynthetically active organisms and light, the nitroxyl radical would couple with the radicals produced in these organisms. If so, the use of a carbon-14

labeled nitroxide would make it possible to isolate and determine which constituent, or constituents, give rise to the photo-induced EPR signals.

The stability of unlabeled DTBN with various organic materials, both in the light and in the dark, was tested. It undergoes reactions in the dark with tetracyanoethylene, p-chloranil, o-chloranil and sodium ascorbate, the rates of disappearance of the nitroxide (ESR signal) being unchanged upon illumination with a tungsten filament lamp. It is inert under both light and dark conditions with N,N-dimethylaniline, N,N,N,N-tetramethylphenylenediamine, quinoline, hexamethylbenzene, hexafluorobenzene, pentafluorobenzonitrile, m-dinitrobenzene, s-trinitrobenzene, lumiflavin, lumichrome, chlorophyll a, 2,6-dichlorophenol-indophenol, 3-(3,4-dichlorophenyl)-1,1-dimethylurea, $K_3Fe(CN)_6$, salicylaldoxime and KBH_4. Cyclohexane, ethanol, chloroform, dimethylformamide, acetonitrile, benzene or the pure material was used as solvent in these experiments. Attempts to demonstrate interaction of DTBN with chlorophyll a were carried out both in cyclohexane and in ethanol. No interaction was observed either in the dark or upon illumination. However, the sdution in ethanol may have been complicated by the presence of traces of oxygen since only a nitrogen purge was used to degas this sample.

Illumination of cyclohexane solutions containing DTBN and various quinones causes a disappearance of the radical, which is very slowly reversible in the dark. The extent of this reversibility is 50 percent or greater. Monochromatic light, of wavelengths absorbed by the particular quinone in the visible region of the spectrum, was used for these illuminations. The quinones capable of effecting this photodestruction of DTBN are 1,4-benzoquinone, 1,4-duroquinone, 1,4-naphthoquinone and 9,10-anthroquinone.

When freshly prepared chloroplasts from spinach leaves are suspended in a 0.5 M sucrose solution buffered at pH 6.8 and containing DTBN, a rapid, irreversible destruction of the nitroxide occurs in the dark. The same observation is made with intact Chlorella cells and with the photosynthetic bacterium Rhodospirillum rubrum. However, when the spinach chloroplasts are allowed to age in a cold room in the dark for a few days, and chloroplast-DTBN mixtures prepared from these aged chloroplasts are examined in the dark, only a slow decay of the nitroxide resonance is observed. When such preparations are illuminated with light of wavelength greater than 5400 Å the rate of destruction of the radical is increased. The rates of both the dark and the light-induced decay vary with the age and treatment of the chloroplast preparations. The same results are obtained with chloroplast fragments obtained by rupturing the chloroplast in 0.05 M sucrose buffered at pH 6.8. Results typical of this effect are shown in Figure 1. We have worked primarily with fragments because the dark reaction is slower with fresh fragments and disappears in fewer days than with the whole chloroplast.

The reduced form of the nitroxide, di-tertiarybutylhydroxylamine (DTBNH) is not oxidized in the dark in the presence of chloroplast fragments. However, if the mixture is illuminated with light of wavelengths greater than 540 mμ, a rapid increase of the nitroxide resonance occurs followed by a gradual decay. The effect is shown in Figure 2. This effect is also observed in samples containing DTBN and freshly isolated fragments, preparations in which 80 to 95 percent of the nitroxide has disappeared during the period between the preparation of the samples and detection of the nitroxide signal in the EPR spectrometer. The change in signal height

is not as large in these latter samples as with the ones containing the deliberately reduced form and fragments. No signal increase is observed with fresh whole chloroplasts with either DTBN or DTBNH, either in the dark or in the light.

The increase in the signal height shown in Figure 2 was shown to be due to an enlarged nitroxide signal and not due to some other photo-generated radical, by sweeping through the three-line spectrum of the nitroxide before and after illumination. Although all samples were purged with nitrogen prior to each experiment, it is conceivable that the increased signal height could be due to a photoreaction involving the uptake of oxygen which would cause a narrowing of the nitroxide resonance accompanied by an increased signal height, if the number of spins remained constant. This is unlikely, however, since the magnitude of the signal change would require a narrowing of the signal by a factor of 2 or more. This narrowing is not observed.

It would appear from these observations that the destruction of the nitroxide in the dark by freshly isolated chloroplast or fragments is a reduction, first to the hydroxylamine and then to some other species probably the secondary amine, which is not photo-oxidized to the nitroxide.

The photo-induced oxidation of DTBNH to DTBN in the presence of chloroplast fragments clearly rules out the possibility that the photodestruction of the nitroxyl radical is a simple reduction to the hydroxylamine.

Considerable information has been obtained about the photo-induced EPR signals in photosynthetic organisms by subjecting them to a variety of environmental treatments. Table I is a resume of the effects of a few of these treatments on the two light-induced free radical signals detected in green materials. Included in this table is the effect of each variation on the photodestruction of the nitroxide.

TABLE I

THE EFFECTS OF ENVIRONMENT ON THE PHOTODESTRUCTION OF DTBN AND SPINACH CHLOROPLAST EPR SIGNALS

Treatment	Broad Signal	Narrow Signal	DTBN Photoreaction
Control	Present	Present	Reaction
64°C - 15 min	Absent	"	Reaction
100°C - 15 min	"	Absent	No reaction
Aged	"	Present	Reaction
DCMU*	Decreased[6]	Enhanced[6]	No reaction
DCPIP*	**	Present	Partial inhibition
Heptane extracted	**	"	" "
Salicylaldoxime	**	"	" "
$K_3Fe(CN)_6$	**	"	" "

* DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol-indophenol.

** Experiments run on aged chloroplast fragments in which this signal is not present.

The chemicals shown in Table I all interact in some way with the electron transfer chain connecting the species participating in the two light acts of photosynthesis. Potassium ferricyanide and the indophenol dye both act as Hill reagents, accepting electrons from different points on the chain. Salicylaldoxime is thought to bind the two copper atoms in plastocyanin, thus terminating electron flow at this point. Heptane extracts the quinones and carotenes. DCMU is a potent inhibitor of photosynthetic oxygen evolution and electron flow in general.

The inhibitory or partial inhibitory effect of these chemical species on the photodestruction of the nitroxide suggests that the nitroxide is coupling with the radical or radicals produced when the fragments are illuminated. If the nitroxide were undergoing a photo-oxidation, and if one assumes that the inhibitory effect of DCME occurs at only one site within the photosynthesizing organism, namely, on the oxygen evolving side of the mechanism, the complete blockade of the photoreaction of DTBN by DCMU would suggest that this photo-oxidation is occurring between the oxidation of water and the first light act. If this were true, however, ferricyanide and the indophenol dye both should cause an increased rate of destruction of the nitroxide. This is not observed.

Furthermore, no absorption due to 2-methyl-2-nitrosopropane dimer can be detected in the ultraviolet spectrum of samples in which the nitroxide radical has been destroyed. The amount of the nitroxyl radical which can be removed photochemically from this system is at least equal to and on several occasions has been several times greater than the total amount of chlorophyll a present in the reaction vessel. This would seem to require that the species with which the nitroxyl radical is reacting is close to

one of the terminals of the photosynthesizing sequence of reactions; a site where materials are present in greater abundance than chlorophyll. The effect of DCMU and the interesting observation of Weaver[8] that large amounts of unbound manganeous ions develop in Chlamydomonas cells grown in the presence of a nitroxide and light, suggest that the site of interaction of DTBN is close to the primary reductant (H_2O).

Further examination of this reaction is being pursued and will be reported in the future. In addition, carbon-14 labeled nitroxide will be used to determine the fate of the nitroxyl radical.

Summary

Preliminary studies of a photochemical reaction between di-tertiary-butylnitroxide and chloroplasts isolated from spinach are reported. The results indicate that the nitroxide is coupling with the photo-induced radicals produced in these photosynthesizing systems. The exact fate of the nitroxide has not yet been determined but is being pursued by the use of a carbon-14 labeled nitroxide, with the object of determining the nature of the photoproduced radical, or radicals, with which it couples.

FIGURE CAPTIONS

Figure 1. The time response of di-tertiarybutylnitroxide (DTBN) EPR signal to light. The initial reaction mixture contained 1.40 mg chlorophyll (a + b) per ml of suspension, 1.67×10^{-4} M DTBN, 0.05 M sucrose, 0.05 M phosphate buffer, pH 6.8. Light with wavelength between 5400 Å and 9800 Å was used. Base line check was made by displacing field from resonance value. Sample purged with N_2.

Figure 2. Effect of light on di-tertiarybutylnitroxide (DTBN) EPR signal in the presence of large excesses of di-tertiarybutylhydroxylamine (DTBNH). The initial mixture contained 1.85 mg chlorophyll (a + b) per ml of suspension, 2.1×10^{-4} M DTBNH, 0.05 M sucrose, 0.05 M phosphate buffer, pH 6.8, N_2 atmosphere, illuminated with light of wavelength 5400 Å to 9800 Å. Check of base line made by displacing magnetic field from resonance. The nitrogen present initially assumed due to air oxidation of DTBNH during sample preparation. The complete oxidation of the DTBNH originally present would result in a signal height of 5.1 units on this scale.

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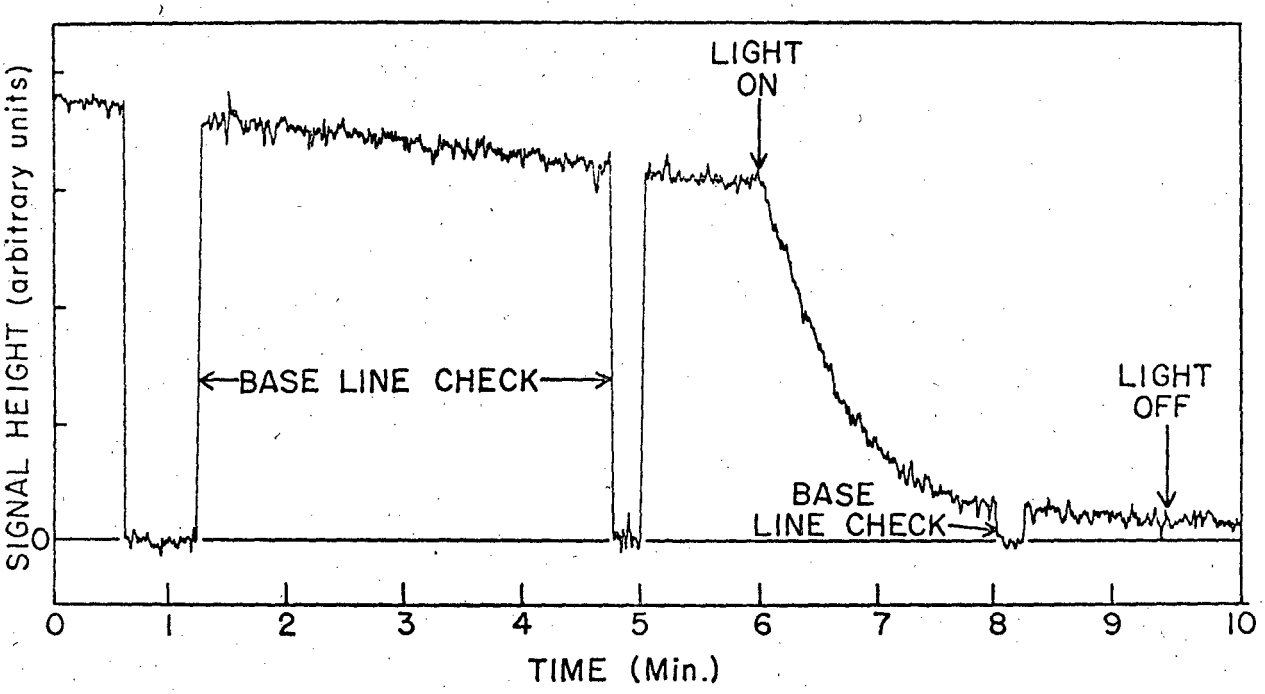
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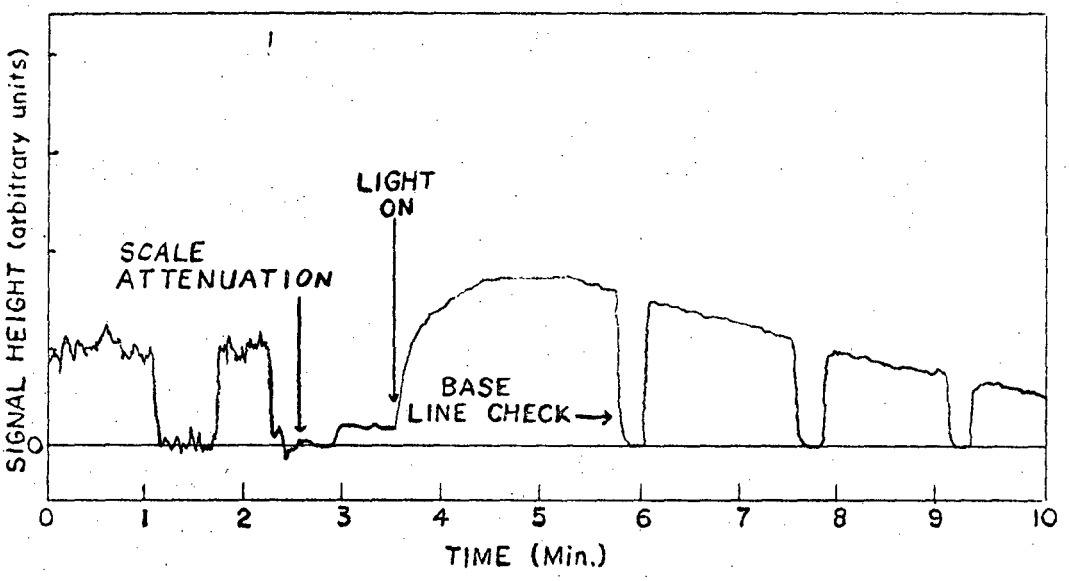
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