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Pulse radiolysis of chlorophyll a in solution

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Pulse Radiolysis
of Chlorophyll a in Solution

by

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<p>15 pages + tables + 8 illustrations</p>	
<p>Abstract</p> <p>Pulse radiolysis of chlorophyll a (Chl a) in pure tetrahydrofuran (THF) and in tetrahydrofuran + 2% triethylamin (TEA) gives rise to a transient electronic absorption band with a maximum at 455 nm. The lifetime and chemistry of this transient strongly depend upon the presence of triethylamin. In pure tetrahydrofuran the transient undergoes a fast second-order decay with a typical half-life of $\sim 8 \mu\text{s}$, and an apparent second-order rate constant $k = 1.5 \cdot 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ is determined. The initial spectrum is replaced by a long-lived residual absorption with maxima at 465 and 480 nm. In tetrahydrofuran + 2% triethylamin the typical half-life is 400 μs, and the products show absorptions at 470 and 520 nm.</p> <p>These results are discussed and the 455 nm transient is tentatively assigned to the radical-anion of Chl a, while the 520 nm product is assigned to β, δ-dihydrochlorophyll a (Krasnovsky's pigment).</p> <p>Available on request from Risø Library, Research Establishment Risø, DK-4000 Roskilde, Denmark. Telephone: (03) 35 51 01, ext. 334, telex: 5072.</p>	<p>Copies to</p> <p>Agricultural Dept. (2)</p> <p>Chemistry Dept. (50)</p> <p>Library (100)</p> <p>Abstract to</p>

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1. INTRODUCTION

Radical-anions of chlorophyll a and related species have been proposed as primary intermediates in the photosynthesis of green plants¹⁾ and bacteria²⁾. Recently, transient absorption spectra assigned to a radical-ion pair have been observed in a bacteria system by application of picosecond flash-photolysis technique³⁾.

Until now only few⁴⁻⁹⁾ studies concerning these radical-anions have been published, and there has been no attempt to carry out systematic studies of the chemical properties. It is our aim to study the radical-anions in vitro using the technique of pulse radiolysis combined with UV/VIS-spectroscopy, resonance Raman spectroscopy and measurements of transient conductivity.

The first part of this study was mainly concerned with the techniques involved in the preparation of the chlorophylls, the selection of the most suitable solvent systems for the pulse radiolysis experiments, and with measurements of the optical spectra of the transients obtained by pulse radiolysis of chlorophyll a solutions. The identity of the various absorbing species is discussed in terms of spectral and kinetic properties. Some preliminary results concerning the chemical reactivity of the transients involved in the chlorophyll a systems were also achieved.

2. EXPERIMENTAL

2.1. Materials

Chl a was prepared from fresh spinach by a combination of several published procedures¹⁰⁾. The procedure used involved chromatography on columns packed with polyethylene and powdered sugar, using, respectively, aqueous acetone and 0.5% n-propanol in isooctane as eluting solvents. The final Chl a solution in isooctane/n-propanol was washed with water several times. Thereby the hydrate of Chl a precipitated. The crystals were isolated by centrifugation and dried in vacuum over solid KOH. The dry product was stored in a freezer. The purity of the Chl a was checked by measuring the ratio of absorbances of the Soret and the red-band maxima using dry diethyl-ether as solvent. Values of this ratio between 1.29 and 1.30 were obtained.

The reagents and solvents used were all analytical grade. All solvents including triethylamin were purified by passing them through a column packed with basic alumina (Woelm) immediately

before use. The diethyl-ether was stored over neutral alumina.

2.2. Samples

All samples were degassed by bubbling with Ar before irradiation. The concentration of Chl a in a particular sample was calculated from the optical density measured at the red maximum, using the published values of the extinction coefficient¹¹⁾.

2.3. Irradiations

All pulsed irradiations were performed at the Risø Febetron 705 B facility. The experimental set-up is shown in Fig. 1. In this part of the work only the conventional UV/VIS detection system based on a xenon lamp and a Hilger Watts 1 m monochromator equipped with a photomultiplier was used. The output from the photomultiplier was digitized on a Biomation transient recorder and the data were stored on magnetic tapes via a PDP-8 mini-computer. All data processing was carried out using the B 6700 computer at Risø.

All irradiations were done using cells with an optical path length of 2 cm. The doses were varied by means of beam-attenuators made of stainless steel diaphragms. All spectra were obtained using an attenuator with an aperture radius of 2.3 mm. This corresponds to an equivalent dose in water of approximately 30 krad.

3. PULSE RADIOLYSIS OF CHLOROPHYLL A

3.1. Alcoholic Solvents

The first experiments were carried out at room temperature on Chl a dissolved in ethanol and 2-propanol. The spectral and kinetic features show that several transient absorbing species are involved in a rather complex reaction mechanism. Within the first 4 ms after the electron pulse at least four mutually dependent transients were detected in the range 450-600 nm. Possibly this complexity is caused by the protic solvents used, giving rise to protonation of the transients formed primarily. This is

supported by the observation that some of the reactions involved are slowed down by the addition of triethylamine (TEA). Because of the complex reaction mechanisms in these solvents, this line of work was discontinued and a search for more inert solvent systems was initiated.

3.2. Tetrahydrofuran

Tetrahydrofuran (THF) is a cyclic ether (C_4H_8O) which, at least in its electronic ground-state, is completely aprotic. Furthermore, it is easily purified and dried by passing it through a column packed with basic alumina. The electronic spectrum of Chl a in THF is shown in Fig. 2. Pulse radiolysis of Chl a in THF gives rise to a fairly strong transient absorption with $\lambda_{max} = 455$ nm as shown in Fig. 3., where normalized absorbance 0.7 ns after the pulse is plotted versus wavelength. This transient relaxes on the millisecond time-scale, as shown in Fig. 4, into a spectrum with $\lambda_{max} = 465$ nm and $\lambda_{max} = 480$ nm. The decay of the transient absorption measured at 460 nm appears to be biphasic, i.e. it consists of a fast and a slow component. The slow decay appears only at time-scales of 2 ms and longer, but it has not been studied in detail.

The half-life of the fast component measured at 460 nm varies with dose between ~ 5 and ~ 40 μ s. Computer analysis of the decay curves indicates second-order kinetics as shown in Figs. 5 and 6. The second-order half-life of a transient absorption is given by

$$\tau_{\frac{1}{2}} = \frac{\epsilon \cdot l}{2kA_0},$$

where ϵ , l and A_0 are extinction coefficient, optical path-length and initial absorbance, respectively. A plot of $\tau_{\frac{1}{2}}^{460}$ versus $1/A_0^{460}$ is shown in Fig. 7. The plot is linear at high doses (i.e. high initial absorbances) as expected for a second order reaction. From the slope of the linear part, we find:

$$2k = \frac{\epsilon^{460}}{3.45} \cdot 10^7 \text{ M}^{-1} \text{ s}^{-1} \quad (l = 2 \text{ cm})$$

Using the value $\epsilon^{460} = 5.3 \cdot 10^3$ calculated later, we find:

$$2k = 1.5 \cdot 10^{10} \text{ M}^{-1} \text{ s}^{-1}$$

This high rate constant is consistent with a decay of the transient by diffusion-controlled recombination between positive and negative ions generated during the electron pulse. Such reactions should follow second-order kinetics because the cations and anions are generated in equivalent amounts.

3.3. Tetrahydrofuran + Triethylamin

The electronic spectrum of Chl a dissolved in THF + 2% TEA is identical to the spectrum in pure THF as far as the values of λ_{max} and ϵ_S/ϵ_R are concerned. If the added TEA has any influence, a blue-shift of both the Soret band and the red band as well as an increase in the ratio ϵ_S/ϵ_R would be expected.

The addition of 2% TEA to the Chl a solution in THF changes, however, both the kinetics and the chemistry of the transients obtained by pulse radiolysis.

The initial spectrum obtained 0.8 μs after the pulse is shown in Fig. 8. Within the first 10 μs a formation is observed in the range 480-540 nm. This formation corresponds to the difference spectrum shown in Fig. 9 where

$$\Delta A(\lambda) = \frac{1}{A_{\text{max}}^{455}} (A(\lambda, t = 15.7 \mu\text{sec}) - A(\lambda, t = 0.8 \mu\text{sec}))$$

is plotted versus λ . It should be noted that this initial formation is only observed when TEA is present.

The primary transient has $\lambda_{\text{max}} = 455 \text{ nm}$, which is identical to the value obtained in pure THF. The transient relaxes to a spectrum with $\lambda_{\text{max}} = 470 \text{ nm}$ and $\lambda_{\text{max}} = 520 \text{ nm}$ as shown in Fig. 10. These spectra are quite different from those obtained in pure THF-solutions, and the change is not caused by the higher concentration of Chl a. Figure 11 shows the spectra obtained from a solution with an approximately five times lower Chl a concentration.

The spectral changes observed in the range 600-1000 nm are shown in Fig. 12. With the concentration of Chl a used in this experiment, it was impossible to measure in the range 640-670 because of the strong absorptions of ground-state Chl a.

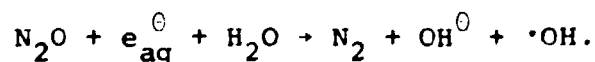
In addition to the dramatic change in chemistry of the primary transient upon the addition of 2% TEA to the THF-solution, an

increase in half-life measured at 460 nm was observed. The half-life of the transient obtained from 19.6 μM Chl a in pure THF with a dose-attenuator of 2.3 was 8 μs , while the transient obtained from a solution of 25 μM Chl a in THF + 2% TEA had a half-life of 400 μs . The initial absorbances were 0.109 and 0.113, respectively, in these experiments. Thus the initial concentrations were almost equal. A typical kinetic plot of normalized absorbance versus time at 460 and 530 nm is shown in Fig. 13. This plot shows that the product absorbing in the range 515-535 nm is formed simultaneously with the decay of the 455 nm transient. At high doses, the decay in THF + 2% TEA appeared to be of second order, but further experiments concerning the details of the kinetics are needed before any conclusions can be drawn.

3.4. Extinction Coefficients

As mentioned above, the decay of the "455 nm" transient is of second order. In order to calculate the rate constants it is therefore necessary to know the extinction coefficient of the transient at the proper wavelength. Such a determination can be made in several ways. The two standard methods are:

- a) Dosimetry with the $\text{K}_4\text{Fe}(\text{CN})_6$ dosimeter. This dosimeter consists of an aqueous solution of $\text{K}_4\text{Fe}(\text{CN})_6$ saturated with N_2O . By pulse radiolysis, all hydrated electrons are converted to OH-radicals:



The $\cdot\text{OH}$ -radicals formed oxidize $\text{Fe}(\text{CN})_6^{4-}$ to $\text{Fe}(\text{CN})_6^{3-}$, which can be measured spectrophotometrically. By use of the relation

$$G(\text{Fe}(\text{CN})_6^{3-}) = G(\cdot\text{OH}) + G(e_{\text{aq}}^{-}),$$

the absorbed doses can be calculated.

In order to use such a value in relation to the experiments on THF-solutions, knowledge is needed of the ratio between the absorbed doses in water and THF, as well as the G-values for ion-formation in THF. The ratio

between the absorbed doses can be calculated from the densities of the solvent, but the G-values for THF have only been measured in a few cases. Furthermore, these G-values are likely to be dependent upon both the dose rate and the nature and concentration of the solutes used.

- b) The product of dose and the G-value for formation of solvated electrons in an organic solvent can be determined from the transient absorption of a known radical-ion. Normally, biphenyl is used as starting material because the corresponding radical-anion can be made under stable conditions by reacting biphenyl with alkali metals. If, however, the solvent system is changed, as is the case in this study, then the extinction coefficient of the biphenyl radical-anion has to be redetermined every time the solvent system is changed.

Because of the problems involved in the use of the above methods, a method giving an absolute determination of the transient extinction coefficient in the Chl a system was found desirable. It is clear that total transformation of the starting material into transient would give immediate information about the extinction of the transient relative to that of the starting material. This method has been used in a flash photolysis study of the triplet state of Chl b¹²⁾, but apparently it has never been applied to pulse radiolysis; probably because of the high doses required. The accelerator, a Febetron 705 B, used in this study is able to deliver doses up to ~1 Mrad. This corresponds to transient concentrations of approx. 1 mM ($0.5 < G < 5$) in most cases.

The experiment is very simple in that it consists of a number of pulses on a given solution with increasing doses until the limiting transient absorption is reached. The interpretation is based on the following assumptions

- 1) The chemical nature of the transient formed is independent of dose
- 2) The constant concentration of starting material is the limiting factor to the high dose plateau of the

transient absorption.

The results from a preliminary experiment on a solution of Chl a in pure THF are shown in Fig. 14. The optical density of the Chl a solution at the red maximum was 1.61 (path length = 1 cm). From this can be calculated an uncorrected value of $4.1 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for the transient at $\lambda = 460 \text{ nm}$. The reported¹¹⁾ value of the extinction coefficient of Chl a in THF has been used. The calculated value for the transient should be corrected for the extinction of Chl a, which is $\sim 1.2 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ at 460 nm. This gives

$$\epsilon_{460}^{\text{transient}} = 5.3 \cdot 10^3 \text{ M}^{-1} \text{ cm}^{-1}$$

Besides the uncertainties from the method itself, this value also contains an uncertainty from the correction for extinction of Chl a. This correction is based on relative wavelength determinations on two different instruments, i.e. a Cary 16 spectrograph and the Hilger Watts monochromator used in the transient detection system. The absorption of Chl a rises very steeply below 460 nm, which means that an uncertainty of just a few nanometers can result in a relative uncertainty on the extinction coefficient used of up to $\sim 100\%$.

4. DISCUSSION

The initially formed transient absorption with $\lambda_{\text{max}} = 455 \text{ nm}$ observed in these experiments is tentatively assigned to the radical-anion of Chl a. A priori, several assignments are possible, namely: the radical-anion, the radical-cation, the triplet state.

The radical-anion could be formed by a reaction between the secondary solvated electrons created by the electron pulse and the ground-state Chl a. The radical-cation could be formed by a reaction between the solvent (i.e. THF) radical-cations and the ground-state Chl a. The mechanism of formation of excited states (of solutes) in THF is not known with certainty, but it has been proposed that the excited solute states could be formed by energy transfer from solvent excited states or by direct excitation by means of the Cerenkov radiation emitted during the electron pulse¹³⁾.

The radical-cation of Chl a cannot be responsible for the observed transient absorptions for several reasons. The electronic spectrum of Chl a⁺ is known in a CH₂Cl₂ solution¹⁴⁾, and the spectrum shows no maxima in the range 400-600 nm, although increased absorption is observed. Furthermore, a dramatic decrease in yield on going from pure THF to THF + 2% TEA would be expected if the cation was involved. In pure THF the solvent cations could react with the Chl a and form the Chl a⁺ species, but this reaction is unlikely in the presence of TEA because of rapid proton transfer from C₄H₉O⁺ to the amine.

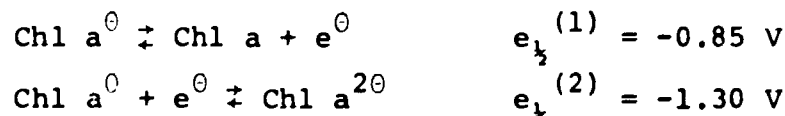
The excited triplet state of Chl a cannot be excluded on the basis of the spectra alone. The triplet-triplet absorption spectrum of Chl a in pyridine shows a maximum around 460 nm¹⁵⁾. The band, however, is very broad and another broad maximum is observed around 550 nm. Although the transient spectra obtained in this work only show one sharp maximum at 455 nm with no significant initial increase in absorption around 550 nm, the possibility of solvent-induced changes of the spectra cannot be excluded.

The changes in chemistry and the increase of the half-lives of the transient observed upon the addition of TEA are, however, not consistent with an assignment of the 455 nm transient to the triplet state. The effect of added TEA suggests that the transient is a charged or strongly polarized species, which is a stronger base than the ground-state Chl a molecule. The effect of bases on Chl a in its lowest triplet state has not been studied, but in general the acid-base properties of triplet states of large organic molecules do not much differ from the acid-base properties of the ground state¹⁶⁾.

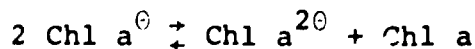
An assignment of the 455 nm transient to the radical-anion of Chl a is supported by the spectral as well as the chemical properties of the transient. In earlier studies concerning the photo-reduction of Chl a by ascorbic acid in pyridine^{4,7)} and the pulse radiolysis of Chl a in THF⁸⁾, transient absorption at 475 and 460 nm, respectively, was tentatively assigned to the radical-anion of Chl a. The wavelength shift from pyridine to THF in the transient difference spectra correlates with the corresponding blue-shift of the Soret band on going from pyridine to THF.

The photo-reduction of Chl a in the presence of bases leads to the formation of the so-called "Krasnovsky pigment"¹⁷⁾. This species has an absorption maximum at 523 nm (pyridine), but it is very unstable and readily reoxidized to Chl a. Recently it was shown that the pigment is the β, δ -dihydrochlorophyll a¹⁸⁾ that is a doubly-reduced Chl a molecule with the π -electronic system in the porphyrin skeleton broken.

In view of these findings, the peak at 520 nm in the final spectra (Figs. 9 and 10) can be assigned to the "Krasnovsky pigment". This assignment is supported by the fact that the formation of the 520 nm product is dependent on the presence of triethylamine. Furthermore, the kinetic traces in Fig. 12 show that the 520 nm product is formed simultaneously with the decay of the 455 nm transient. Therefore, the 455 nm transient must be the precursor of the 520 nm product. This fact supports the assignment of the 455 nm peak to the radical-anion, if the mono-anion can disproportionate to the di-anion. From the published data⁶⁾ concerning the electrochemical reduction of Chl a in dimethylsulphoxide, an equilibrium constant of $2.5 \cdot 10^{-8}$ for the disproportionation of Chl a⁰ to Chl a²⁰ can be calculated as follows:



giving for



$$E^{\ominus} = -1.3 - (-0.85) \text{ V} = -0.45 \text{ V}$$

and

$$K = \frac{[\text{Chl a}] [\text{Chl a}^{2\ominus}]}{[\text{Chl a}^{\ominus}]^2} = 10^{-\frac{0.45}{0.059}} = 2.5 \cdot 10^{-8}$$

If we accept this value as a measure of the equilibrium in pure THF, a value of $[\text{Chl a}^{2\ominus}]_{\text{eq}} \approx 10^{-14}$ M for

$$[\text{Chl a}]_{\text{eq}} \approx 2 \cdot 10^{-5} \text{ M and } [\text{Chl a}^{\ominus}]_{\text{eq}} \approx 10^{-6} \text{ M}$$

can be calculated.

This means that the dianion would not be formed in detectable amounts in an equilibrium mixture. In a real system protonation would, of course, interfere. Protonation of the dianion would drive the equilibrium towards the right and increase the yield of the doubly-reduced product. However, such effects should be more important in pure THF than in THF + 2% TEA, contrary to experimental findings. Rapid protonation of the radical-anion could prevent the formation of the doubly-reduced product in pure THF. Such a protonation should, however, be detectable in the spectra but it is not found.

The effect of amine on the photo-reduction of Chl a, as well as the reactions of the 455 nm transient observed in this work, could possibly be explained by specific interactions between Chl a and the amine altering the one- and two-electron reduction potentials as well as the equilibrium constant for disproportionation. That the equilibrium constant changes upon the addition of amine is suggested by the upper limit for the rate constant for the forward reaction



$$k_f \lesssim 10^2 \text{ M}^{-1} \text{ s}^{-1}$$

which can be calculated from the equilibrium constant mentioned above. This low rate constant can never explain the observed half-life of $\sim 350 \mu\text{s}$ for the formation of the 520 nm product.

In conclusion it is therefore suggested that the initially formed transient is the radical-anion of Chl a in pure THF, as well as THF + 2% TEA. In pure THF, the radical-anion undergoes rapid reoxidation and protonation followed by further unknown reactions. In THF + 2% TEA the radical-anion (or some specially solvated analogue) undergoes disproportionation followed by protonation of the dianion formed, leading to dihydrochlorophyll a.

4.1. The Future

The work described above suggests a series of experiments needed to clarify the reactions involved in pulse-irradiated Chl a solutions. The most important are:

- 1) Further studies concerning the effect of Chl a concentration, TEA concentration and irradiation doses.
- 2) Measurement of the transient conductivity in the system after pulse irradiation. These experiments will be performed at the Royal Technical University of Stockholm.
- 3) Electrochemical studies concerning the effect of amine and other bases on the reduction-potentials of Chl a.

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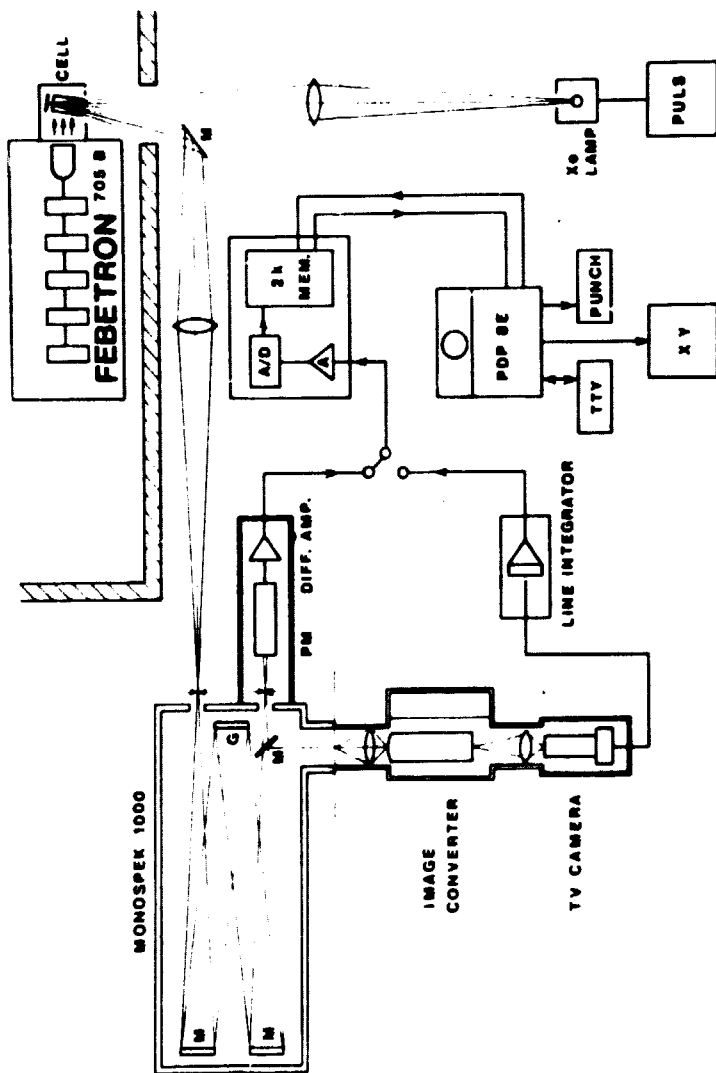


Fig. 1. Diagram of the pulse radiolysis instrumentation.

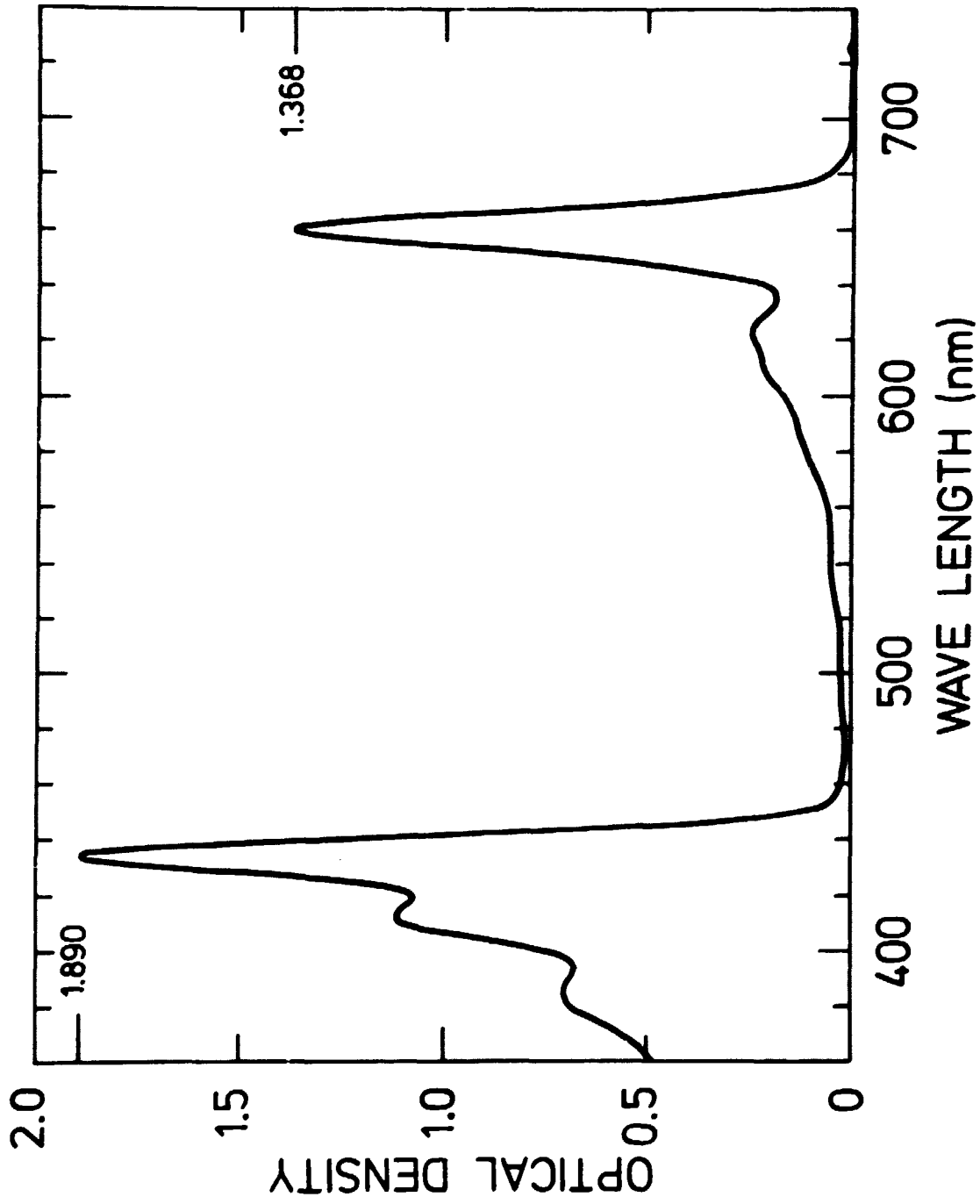


Fig. 2. Electronic absorption spectrum of Chl a in THF.

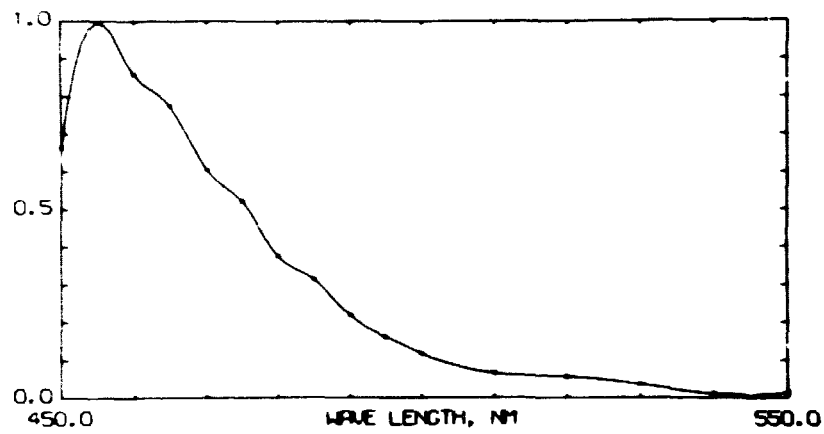


Fig. 3. Normalized transient spectrum, 0.7 μ s after electron pulse. Maximum absorbance = 0.117 [Chl a]₀ = 19.6 μ M.

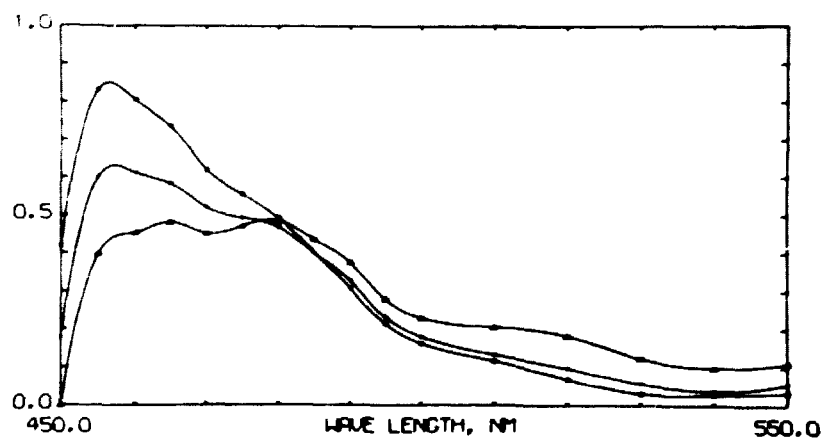


Fig. 4. Normalized transient spectra. Pure THF.
X = 8 μ s + = 40 μ s Δ = 920 μ s
after electron pulse.
Normalized relative to A = 0.092
[Chl a]₀ = 19.6 μ M

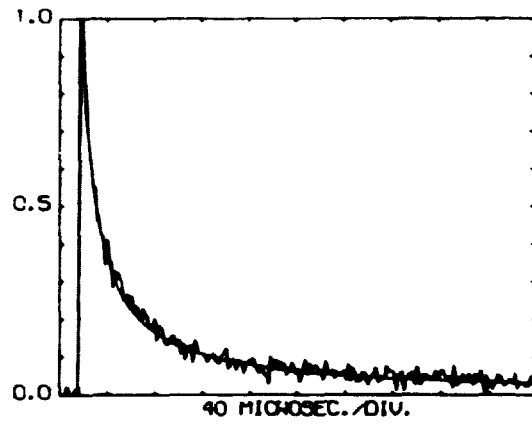


Fig. 5. Decay curve at $\lambda = 460$ after pulse with relative dose of 1.44. The figure also shows the least-square fitted analytical second order curve.

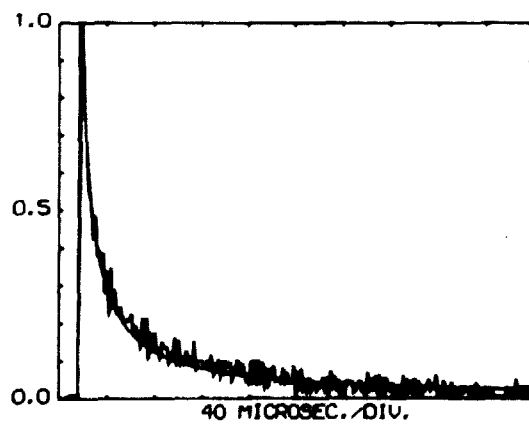


Fig. 6. Same as Fig. 5, but with relative dose of 5.29.

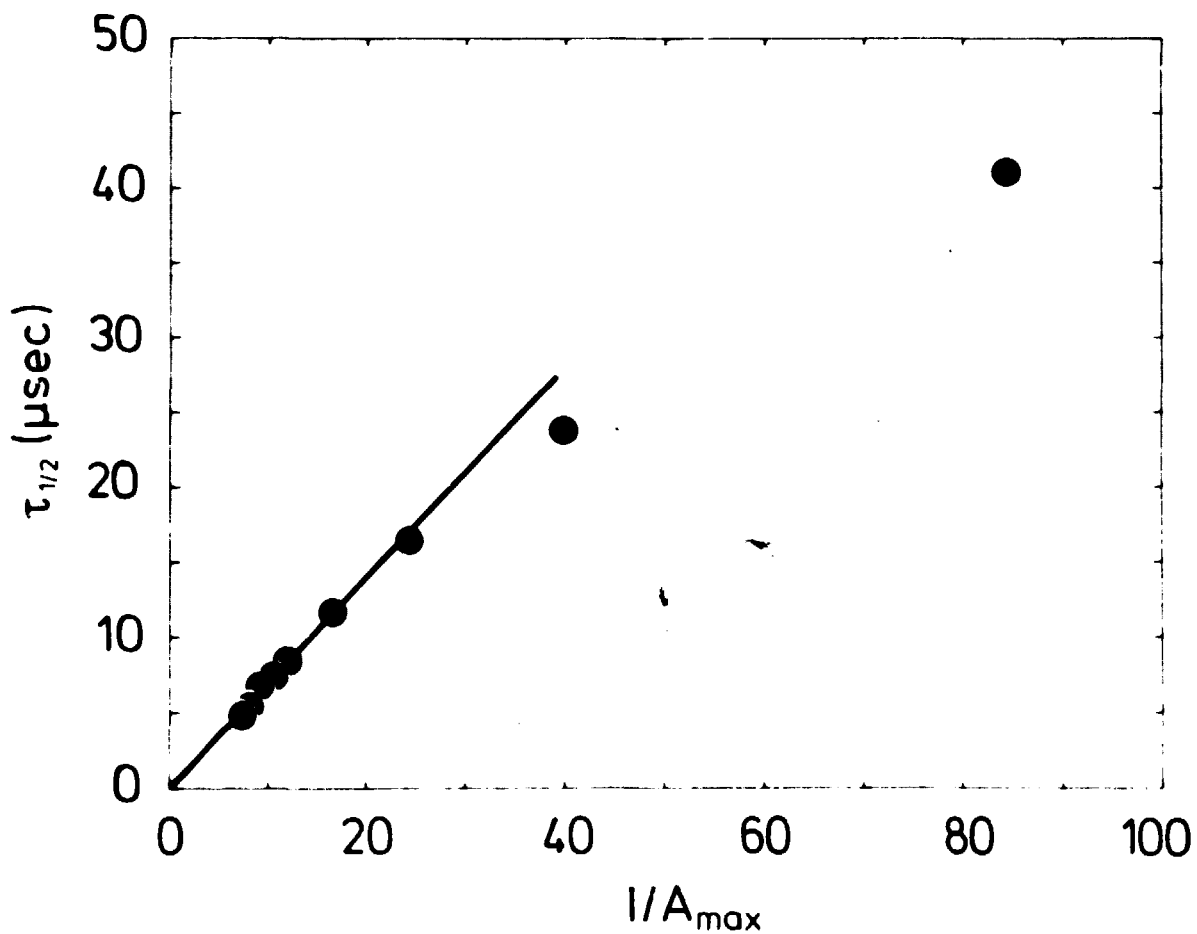


Fig. 7. Plot of $\tau_{1/2}^{460}$ versus $1/A_{\text{max}}^{460}$ for the transient obtained in pure THF from solution 19.6 μM in Chl a.

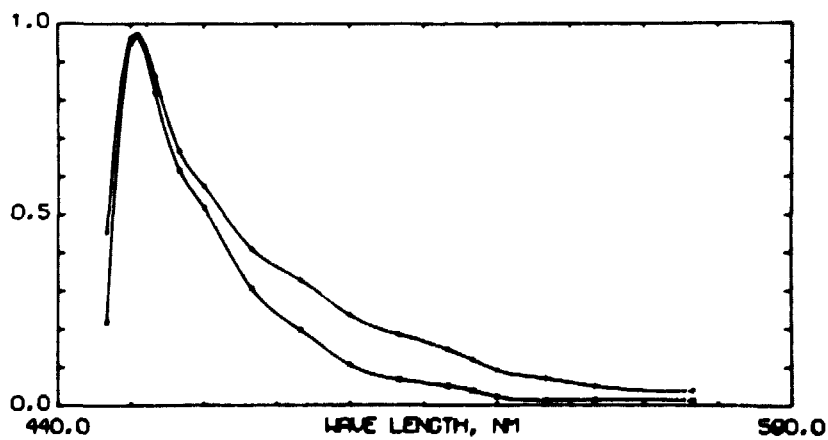


Fig. 8. Normalized transient spectra THF + 2% TEA.
X = 0.8 μs + = 15.7 μs
after electron pulse. $[\text{Chl a}]_0 = 120 \mu\text{M}$.
Normalized relative to A = 0.490.

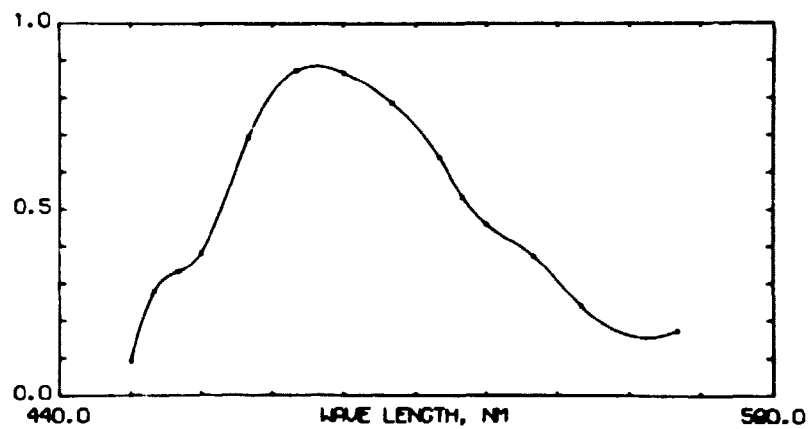


Fig. 9. Difference spectrum between the spectra shown in Fig. 8.

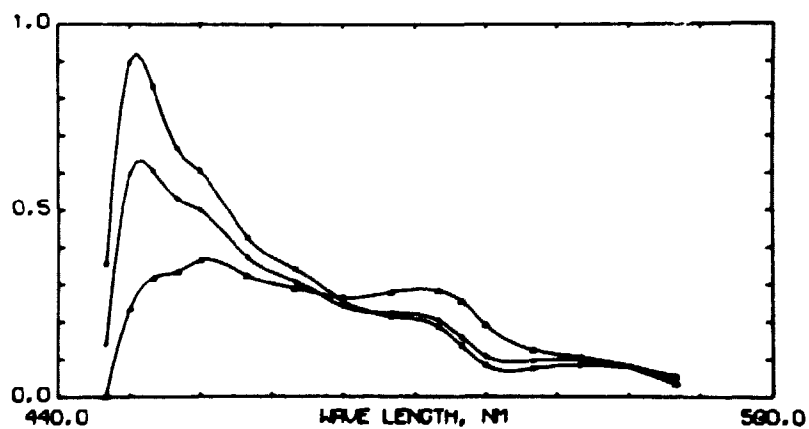


Fig. 10. Normalized transient spectra. THF + 2% TEA.
X = 32 μ s + = 192 μ s Δ = 3680 μ s
after electron pulse.
Normalized relative to A = 0.460. [Chl a]₀ = 120 μ M.

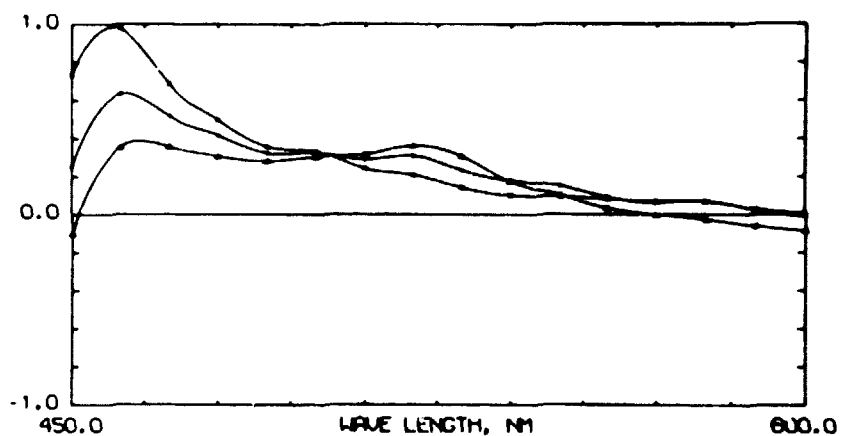


Fig. 11. Normalized transient spectra. THF + 2% TEA.
X = 32 μ s + = 480 μ s Δ = 3680 μ s
after electron pulse.
Normalized relative to A = 0.113. [Chl a]₀ = 25 μ M

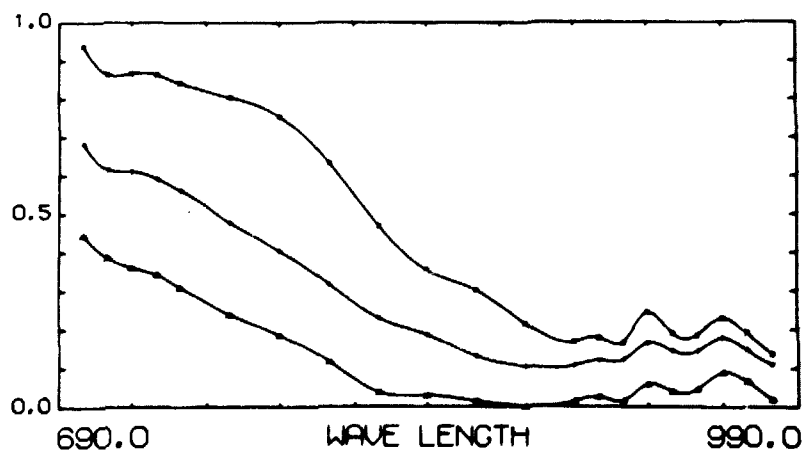


Fig. 12. Normalized transient spectra. THF + 2% TEA.
X = 25 μ s + = 361 μ s Δ = 1881 μ s
after electron pulse.
Normalized relative to A = 0.177 [Chl a]₀ = 65 μ M

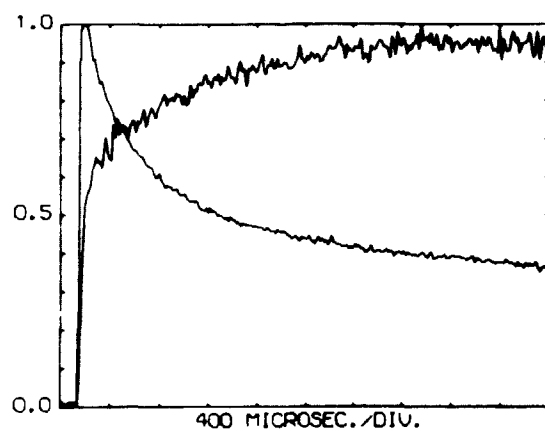


Fig. 13. Decay at 460 nm and formation at 530 nm after pulse radiolysis of Chl a in THF + 2% TEA. Relative dose 5.29. $[\text{Chl a}]_0 = 25 \mu\text{M}$.

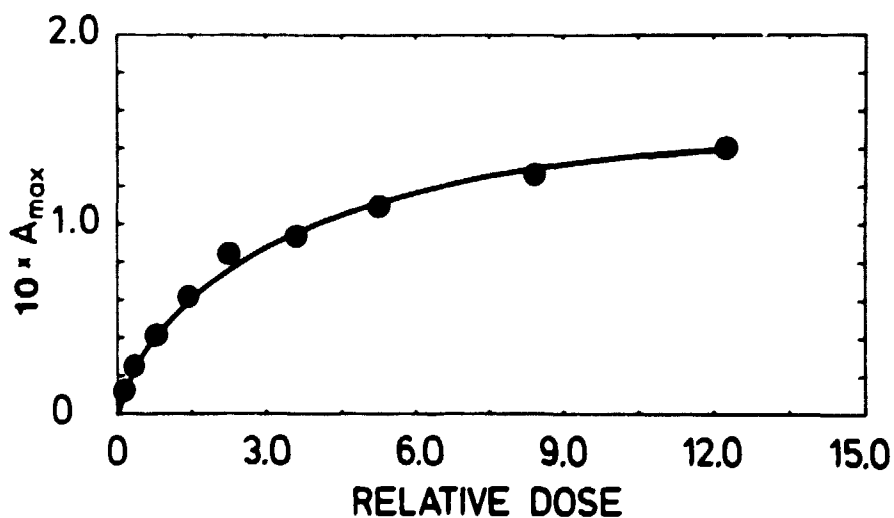


Fig. 14. Plot of A_{max}^{460} versus relative dose for $[\text{Chl a}]_0 = 19.6 \mu\text{M}$ in pure THF.

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