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REACTION OF CHLOROPHYLL WITH 2,2'-DITHIOBIS(5-NITROPYRIDINE)

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Abstract.--Chlorophyll a reacts photochemically with 2,2'-dithiobis(5-nitropyridine), incorporating 5-nitropyridinethiyl residues. On nmr evidence, substitution occurs principally at the α and δ meso positions, and not at the β. The products are markedly less fluorescent than chlorophyll itself, especially in polar solvents, and it is proposed that fluorescence is quenched by electron transfer to the nitropyridine residue.

1. Introduction

In recent papers, the photoreduction of nitroaromatic compounds by chlorophyll a (Chl) on particles of polyethylene swollen with tetradecane, with hydrazobenzene as the "sacrificial reductant" has been described (1,2). All of the nitro compounds were reduced under these conditions; in some cases, passage of the reduction product into the aqueous suspending phase, leaving azobenzene in the particle, achieved separation of the products. Among nitro compounds susceptible to photoreduction is the familiar reagent for protein sulfhydryls, 5,5'-dithiobis(2-nitrobenzoic acid), which is reduced to the strongly colored nitrophenylthiolate anion (3).

With the intention of using this reduction as a convenient test for efficacy of reductants in this reaction system, this compound and the related sulfhydryl reagent, 2,2'-dithiobis(5-nitropyridine) (DTNP), were tested, the latter having somewhat better solubility properties in the context. Indeed, photoreaction of DTNP with hydrazobenzene, sensitized by Chl in a particulate system showed rapid and smooth buildup of absorption in the near-uv region attributable to the expected azobenzene and nitropyridinethiolate anion (Fig. 1). This, and all reactions described, were run under cover of N_2.

Because of recent interest in the possibility that tyrosine residues might act as primary reductants in Photosystem 2 of photosynthesis (4), the next reductant tested was tyrosinamide, with the result shown in Fig. 2. There is a limited increase of absorption in the near-uv region as expected for the nitropyridinethiolate anion, but also a broadening and red shift of the Chl band totally absent in the hydrazobenzene case. The tyrosinamide band, further into the uv, appeared unaffected by the reaction. A control experiment without tyrosinamide produced exactly the same spectral changes. Besides showing that tyrosinamide is not a reductant for the Chl^+ under these conditions, this experiment revealed a new reaction in which Chl apparently serves as a reductant for DTNP.

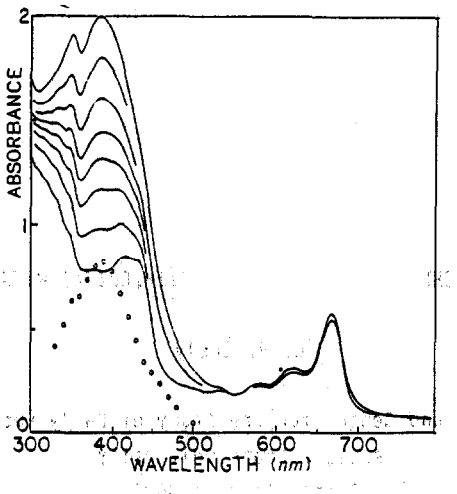


Fig. 1. Photoreduction of DTNP (0.8 mmolal) by hydrazobenzene (18 mmolal), sensitized by Chl adsorbed on polyethylene-tetradecane particles with dodecylhistamine and dodecyltrimethyl ammonium chloride, suspended in a solution of guar gum with cellulose as light scatterer, and irradiated for a total of 500 s by a focused projection lamp beam passed through a 670 nm interference filter. Spectra were recorded after (from bottom) 0, 25, 50, 75, 100, 150, 250 and 500 s irradiation. The 0-150 s difference spectrum (circles) shows nitropyridinethiolate (388 nm) and azobenzene bands.

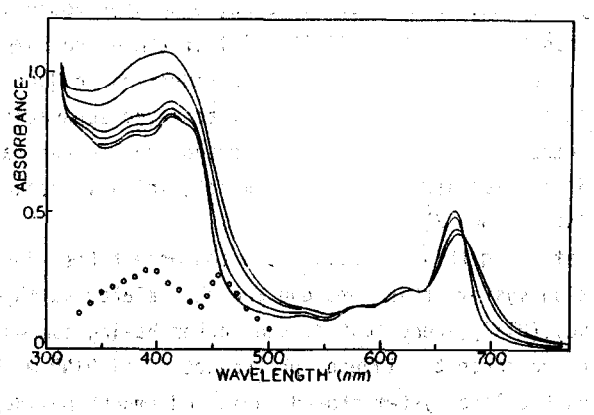


Fig. 2. Composition of reaction mixture is similar to that of Fig. 1 except for tyrosinamide instead of hydrazobenzene. Irradiation for 0, 100, 500 and 1000 s through a 670 nm interference filter (increasing absorbance except at Chl peaks), and for 500 and 1000 s through orange filter #3484. The difference spectrum (beginning to end) is broad and shows a blue shift of the Chl Soret band.

It was noticed that the fluorescence of Chl faded during the reaction and at the end was quite faint. A quantitative determination of fluorescence according to our usual procedures (5) led to values of .013 to .027, depending on wavelength of excitation (430, 450, or 620 nm). Fluorescence spectra were broad, and at 450 nm excitation, the peak wavelength was 689 nm.

A provisional account of events during this reaction is presented in the following equations:

$$^{T}Chl + DTNP = Chl^{+} + DTNP^{-} \quad (1)$$

$$DTNP^{-} = \cdot SPyNO_2 + {}^{-}SPyNO_2 \quad (2)$$

$$Chl^{+} + \cdot SPyNO_2 = ChlSPyNO_2^{+} \quad (3)$$

$$ChlSPyNO_2^{+} + B = ChlSPyNO_2 + BH^{+} \quad (4)$$

where ^{T}Chl is the triplet state of Chl, and B is any base, such as water or the thiolate anion. Other sequences leading to the same products can be envisaged, but this appears to be the simplest. The reaction does not appear to be one of high quantum yield, and simple reversal of steps (1) and (2) is a much more common outcome.

2. Reaction in Solution.

The reaction between Chl and DTNP in tetradecane proved to be very slow and was complete only after 40 min of direct, unfiltered sunlight. A number of other solvents were tested, using W lamp light filtered through a yellow cutoff filter #3486. The reaction was followed by visible fading of fluorescence and by changes in the absorption spectrum; rates were compared through the absorption increases at 470 and 390 nm.

The qualitative results are set forth in Table 1 and are summarized as follows. The reaction was much faster in polar solvents than in non-polar ones, and was substantially incomplete in tert-butyl methyl ether (mtbe) in the time allowed. The shift in the red band varied with the solvent, and not always in the way one might expect (e.g., pyridine). In alcohols, the reaction went in two distinct stages, with successive isosbestic points, and fluorescence was largely quenched in the first. The most rapid reaction was obtained by adding pyridine to 95% ethanol, presumably supplying the base required by step (4) of the reaction scheme. Evidently the fairly rapid reaction seen in the particulate system (Fig. 2) is taking place at the polar interface of the particles with the suspending medium.

TABLE 1

Spectral and Rate Data for Reaction of Chl (1.4×10^{-5} M) with DTNP (ca. 1.4×10^{-3} M) in Eight Solvents.

solvent[a]	λ_{init},[b] nm	λ_{-flu}, nm	λ_{fin}, nm	$10^3 d(\rho_{470}+\rho_{390})/dt$,[c] s^{-1}
ethanol:pyridine 6.2:1	666.2	669.4	672.2	6.63
dimethylformamide[d]	664.6	664.6	664.6	5.26
pyridine	671.1	669.0	669.0	1.83
ethanol, 95%	665.2	668.6	670.0	1.77
methanol	665.2	668.4	672.1	1.27
acetone	662.4	--	663.4	1.04
1-propanol	664.0	667.7	667.8	0.82
t-butyl methyl ether	662.0	--	(662.0)	0.10

(a) Ranked in order of reaction rate.
(b) Positions of red band peak at start, when fluorescence has visibly faded, and at end of reaction are listed.
(c) Rate of absorbance increase during first light period as measure of rate of nitropyridinethiol formation.
(d) Probably slightly basic.

TABLE 2

Spectral Data and Fluorescence Quantum Yields in Various Solvents for First Fraction from Chl-DTNP Reaction.

Solvent	λ_{abs}, nm[a]	λ_{flu}, nm	ϕ[b]
toluene	667.5 ± 0.5	677.5 ± 0.5	0.092
mtbe	664.5	674.0	0.072
1-propanol	668.5	676.0	0.034
acetone	664.0	671.5	0.024

(a) Columns list wavelengths of peak absorbance and fluorescence, and quantum yield relative to that of rhodamine 101 taken to be 0.96. The peak positions are about 3 nm to longer wavelengths than those of Chl.
(b) Quantum yields are not corrected for reabsorption, which is considerable, but would affect all values proportionately.

Products from the reactions in mtbe, acetone, propanol, ethanol, and ethanol + pyridine were subjected to reverse phase analytical HPLC in a 90-100% methanol (10-0% water) gradient. The profile of eluted products varied somewhat from preparation to preparation. Generally, products emerged in three groups, each appearing to have several components. All had spectra similar to Chl but with red bands at about 674, 670, and 667, in order of appearance, and in inverse order of their formation during the reaction. Chl, if present, appeared among the last group.

The preparations were also subjected to tlc on silica gel, in 9.2:0.8 benzene:1-propanol. The banding correlated well with the results of HPLC.

Fluorescence was further investigated with the first-formed fraction of products of reaction in ethanol-pyridine. Portions were dissolved in toluene, mtbe, acetone and 1-propanol, and fluorescence quantum yields were determined by comparison with rhodamine 101, following established procedures (6). The results, in Table 2, show that this product is more fluorescent in non-polar solvents than in polar ones, confirming casual observations of its behavior. The result is best explained if polar solvents stabilize or promote electron transfer from the Chl residue to the nitropyridinethiyl in the singlet excited state. Later formed fractions appear less fluorescent than the first, whether on the chromatographic column or in solution, indicating that electron transfer in them is more exothermic.

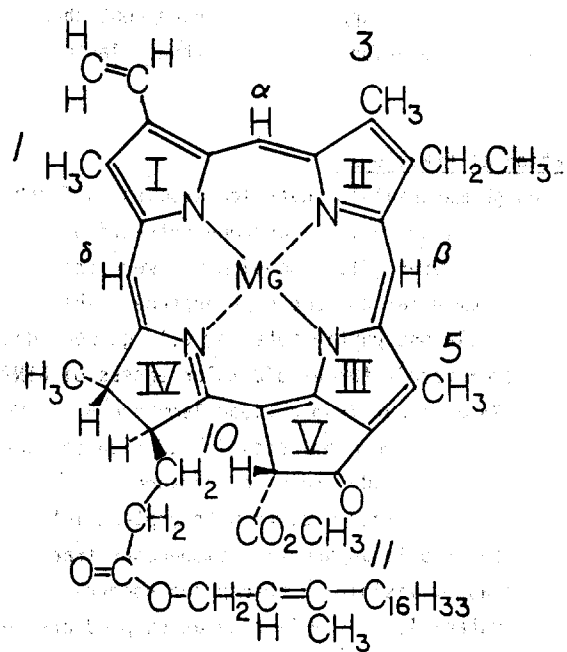
3. Further Characterization of Fractions.

In order to obtain enough material for detailed studies, a fresh sample of Chl was prepared from spinach by dimethyl sulfoxide - acetone extraction and precipitation twice from acetone + 15% dioxane with water (7). The pigments were then put on a short sugar column and washed with petroleum ether to remove β-carotene. The remaining pigments, mainly Chl *a*, Chl *b*, and a little pheophytin totaling 0.10 g, were dissolved in 70 ml of 6:1 ethanol:pyridine, an approximately three-fold molar excess of DTNP was added, and the contents were exposed under N_2 to light from a flood lamp for 6 h. When the reaction was stopped, a tlc test strip showed a number of bands in addition to starting material. The product was recovered by evaporation under reduced pressure.

By repeated chromatography on powdered sugar with heptane, mtbe and propanol, the reaction product was separated into 11 apparently homogeneous fractions large enough to be characterized by nmr as well as by visible-uv absorption spectra. The first six fractions were identified as starting material, or simple alteration products including Chl *b'* and perhaps Chl *b*-3-methanol. Almost all of the Chl *b* and the pheophytin a was recovered unreacted. The remaining five fractions had a Chl *a*-type spectrum, with only a shoulder at 465 nm to indicate the presence of Chl *b* derivatives. The last two fractions could only be eluted from sugar with pure propanol. Fluorescence intensity ranged from weak to almost zero with increasing fraction number. A total of 0.067 g was recovered in these fractions, about two-thirds of the starting material.

Although fractions 7, 8 and 9 appeared homogeneous on the sugar column, tlc on silica gel showed at least 5, 5, and 6 components in them respectively. Fractions 10 and 11 appeared similar and were not further resolved in tlc.

The low-field nmr spectra of these fractions in acetone-d6 are extremely disturbed. The example of fraction 7, which has two main components, is shown in Fig. 3 where it is compared with that of the second fraction, Chl a. The β-H appears intact, but the α-H and δ-H signals are diminished. The vinyl group appears intact, but much distorted. Locations and spacings of several pairs of bands, e.g., at 9.4, 8.4 and 7.75 ppm, suggest their origins in the 6-H, 4-H and 3-H of the nitropyridinethiyl group. In the mid-field region, the 5- and 11-CH_3 protons appear little altered, but change is seen in the 3- and 1-CH_3 region. The high-field region (not shown) is little changed. The appearance of the spectrum is tentatively explained by substitution of nitropyridinethiyl at the α or δ position. Nmr spectra of fractions 8 and 9 are similarly complicated, but those of fractions 10 and 11 are broadened almost into unrecognizability, probably by association even in acetone.



CHL A

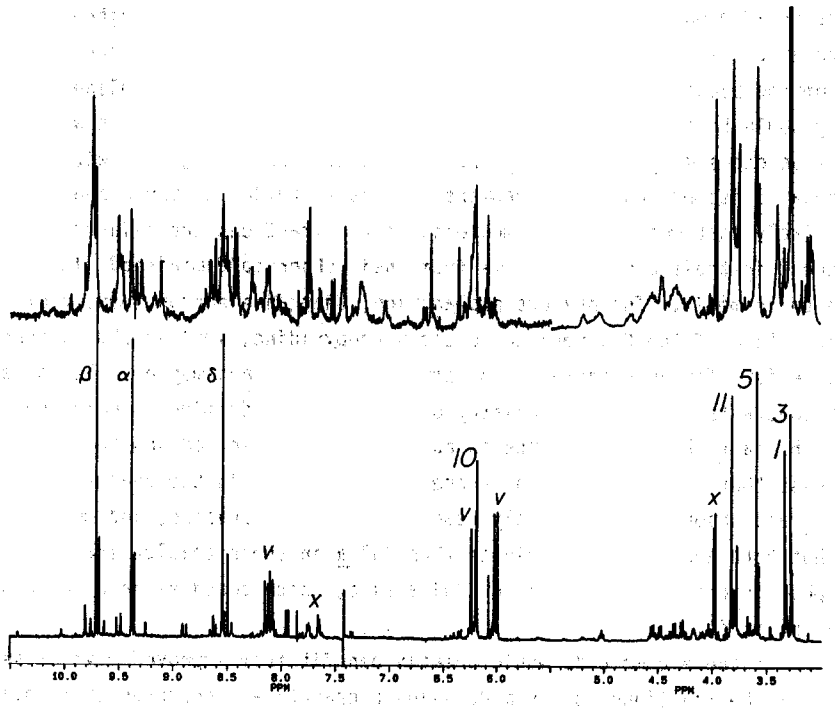


Fig. 3. Comparison of low-field and mid-field ranges of nmr spectra of fraction 7 and fraction 2 (Chl) of large-scale photochemical reaction. Chl bands are assigned as in ref. (9). Bands marked "v" are of the 2-vinyl group, those marked "x" do not belong to Chl. Accompanying most bands in the Chl a spectrum are smaller bands belonging to Chl a'. The region 3.0-5.5 ppm is on a smaller vertical scale than the low-field region.

4. Discussion

Absorption spectra of all of the fractions show clearly that the phorbin chromophore of Chl remains intact. This, and the derangement of the nmr resonances of many of the peripheral substituents impose severe restrictions on the types of structures that can be considered for the products. The nmr suggests substitution at the α and δ positions but not at the β, and does not rule out addition to the vinyl in the higher fractions. The progressive nature of the reaction suggests multiple substitution, but the nmr evidence is too vague to confirm this.

Since the C-S-C bond angle is nearly tetrahedral, substitution of nitropyridinethiyl into a meso position of the crowded Chl ring would swing the pyridine group into an angle almost 70° from the plane. Since rotation of the pyridine through the plane of the porphyrin ring would be extremely difficult, diastereomers should exist in which the substituent is on one side or another of the Chl plane. These, together with epimers at C-10, could total 8 isomers and diastereomers with substitution at the α and δ positions only. It is likely that most of the components of the fractions are related in this way.

Our interest in these compounds stems from their observed diminished fluorescence, especially in polar media. The obvious explanation, that an electron jumps from the singlet excited state of the Chl residue to the nitropyridine, implies that a charge transfer state exists for an unknown period after quenching, and may be able to react with oxidants and reductants. The photochemistry of these compounds might, therefore, be very interesting. The fact that fluorescence is not completely quenched and that it is solvent dependent suggest that a large amount of energy may be stored in the charge-transfer state.

That Chl b and pheophytin a scarcely react is mildly surprising, and must be related to the fact that both are harder to oxidize than Chl a in their triplet states (8). Probably the photoreaction is marginal for Chl a also, which could explain the considerable effect of solvent on it.

Future work on these compounds would greatly benefit from improved ways of separating the components of the fractions, or from devising preparative procedures that obviate formation of some of them.

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