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THE OXIDATION OF CHLOROPHYLL *a* IN ALCOHOLS*

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SUMMARY

Oxidation potentials for the chlorophyll (Chl/Chl$^+$) couple, relative to that of the Fe^{2+}/Fe^{3+} couple, were determined in methanol, ethanol, and isopropanol from the initial extent of reaction of chlorophyll with FeCl$_3$. The presence of 4,7-dimethylphenanthroline was necessary to get sufficient oxidation of chlorophyll in isopropanol. The values were +0.75 V, +0.865 V, and approx. +1.0 V, respectively, based on an assumed value of +0.77 V for the Fe^{2+}/Fe^{3+} potential. It is suggested that alcohols, especially methanol, may stabilize Chl$^+$ by adding to the carbonyl group or the conjugated double bond system.

INTRODUCTION

The oxidation of chlorophyll *a* (Chl) by FeCl$_3$ in methanol was first reported by RABINOWITCH AND WEISS[1]. WATSON[2] established the reversibility of the reaction, and GOEDHEER, HORREUS DE HAAS AND SCHULLER[3] estimated potentiometrically a value of about +0.395 V *versus* the saturated calomel electrode (S.C.E.) for the presumed half-reaction:

$$\text{Chl} \rightleftharpoons \text{Chl}^+ + e^- \qquad (1)$$

The potential of the Chl/Chl$^+$ couple is important to an understanding of photosynthesis, because photochemically produced oxidized chlorophyll species have been implicated as oxidants of unknown substrates both in System I, where NADP$^+$ is reduced[4], and in System II, where water is oxidized[5,6]. Our interest in the matter stems from observations that although chlorophyll is readily oxidized by FeCl$_3$ in methanol, it is oxidized to a much smaller extent or not at all in most other solvents, including ethanol. The differences seemed greater than could be explained merely as an effect of solvent on the Fe^{2+}/Fe^{3+} potential, and suggested that methanol might somehow exert a special stabilizing effect on the oxidized chlorophyll. If this were so, the value for the potential measured in methanol might not be the appropriate one for chlorophyll under physiological conditions.

After a brief survey of other solvents, we were led to a more detailed exami-

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Abbreviations: S.C.E., saturated calomel electrode; DMP, 4,7-dimethylphenanthroline; phen, 1,10-phenanthroline.

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nation of the oxidation of chlorophyll *a* in a series of alcohols: methanol, ethanol, isopropanol and *tert.*-butanol. We chose to avoid potentiometric measurements because of the difficulties of accurately determining liquid junction potentials. The potential for Reaction 1 was calculated from measured values of the equilibrium constant K for the reaction

$$\mathrm{Chl} + \mathrm{Fe}^{3+} \rightleftharpoons \mathrm{Chl}^{+} + \mathrm{Fe}^{2+} \tag{2}$$

$$K = \frac{[\mathrm{Chl}^{+}]\,[\mathrm{Fe}^{2+}]}{[\mathrm{Chl}]\,[\mathrm{Fe}^{3+}]} \tag{3}$$

and an assumed potential for the $\mathrm{Fe}^{2+}/\mathrm{Fe}^{3+}$ couple, by means of the Nernst equation. Anticipating that FeCl_3 might not be strong enough to oxidize chlorophyll appreciably in the higher alcohols, we added 4,7-dimethylphenanthroline (DMP) when necessary to stabilize Fe^{2+} by complex formation. The potential for the half reaction

$$\mathrm{Fe\,(DMP)_3}^{2+} \rightleftharpoons \mathrm{Fe\,(DMP)_3}^{3+} + e^{-} \tag{4}$$

is 0.695 V (S.C.E.) in water[7]. Unfortunately, the instability of alcoholic solutions of $\mathrm{Fe\,(DMP)_3}^{3+}$ made direct addition of this oxidant impossible.

MATERIALS AND METHODS

Chlorophyll *a* was prepared from spinach by the method of ANDERSON AND CALVIN[8]; 4,7-dimethylphenanthroline was purchased from the J. T. Baker Chemical Co. Ethanol (Commercial Solvents Hexagon Grade 95 %), methanol and isopropanol (Mallinkrodt Analytical Reagent) were used as received, but *tert.*-butanol was frozen repeatedly until it was entirely solid at 25°. Oxidation was performed by injecting 5–100 μl of a 10^{-3} M solution of $\mathrm{FeCl_3 \cdot 6\,H_2O}$ in the appropriate alcohol through a Teflon needle into a cuvette containing a solution of chlorophyll, and DMP when appropriate, in the alcohol. The cuvette, of 5-mm path length and about 4 ml capacity, was held in the sample compartment of a Cary 14 Spectrophotometer, and flushed continuously with nitrogen (Matheson Co., Prepurified Grade) to expel oxygen and mix the contents. Since the reversible oxidation product of chlorophyll more or less rapidly undergoes secondary, irreversible reactions, the absorption band of chlorophyll was followed continuously and the absorbance was extrapolated back to the time at which the contents were half-mixed (5–10 sec after addition of $\mathrm{FeCl_3}$). The nitrogen bubbles were not in a position to interfere with the absorbance measurement. The temperature was 24°.

The chlorophyll concentration was usually about $2.5 \cdot 10^{-5}$ M. The Fe^{2+} concentration after reaction was calculated from the change in absorbance at the chlorophyll band near 665 nm (the absorptivity of Chl^{+} at that wavelength is quite low), taking into account that the $\mathrm{FeCl_3}$ initially contained a small amount (about 1 %) of Fe^{2+}. The Fe^{2+} content of a solution was occasionally determined directly by conversion to the DMP complex.

RESULTS

In a preliminary survey of the effect of solvent on oxidation of chlorophyll, $\mathrm{FeCl_3}$ was added to $2 \cdot 10^{-5}$ M chlorophyll in various solvents and the change in absorbance of the band near 665 nm noted. In dioxane, *n*-butanol, dimethylsulfoxide, *tert.*-butanol, and acetonitrile, there was no detectable reduction in absorbance in the presence of $5 \cdot 10^{-4}$ M $FeCl_3$. In ethanol and dimethylformamide there was about 10% reduction. In acetone, *sec.*-butanol, and chloroform there was about 100%, 75%, and 50% reduction at $5 \cdot 10^{-4}$ M $FeCl_3$, and in methanol there was a 50% reduction at $2 \cdot 10^{-5}$ M $FeCl_3$. The solvents were not specially purified, and impurities in them (particularly in *sec.*-butanol and chloroform) may have been responsible for the bleaching, or the lack of it. These results indicated that a detailed examination of oxidation of chlorophyll in a series of alcohols might be instructive. The oxidation of chlorophyll in acetone was noted by RABINOWITCH AND WEISS[1], and merits further examination.

Methanol. Oxidized chlorophyll is relatively stable in absolute methanol, and chlorophyll is almost quantitative regenerated if a reducing agent such as hydrazobenzene or ascorbic acid is added within a few sec after the addition of $FeCl_3$. If no reducing agent is added, and if the amount of $FeCl_3$ is small, chlorophyll is gradually regenerated, with a half-time of about 10 min, as Fe^{3+} is reduced by methanol or possibly an impurity in it. If the amount of $FeCl_3$ is large, the red band in the spectrum of the regenerated pigment is at 671 nm instead of 665 nm, its position for chlorophyll.

Initial absorbance changes were determined by linear extrapolation back to the time of half-mixing. The scatter in the values of K (Table I) reflects the difficulty inherent in measurements of this unstable system. The difference between the standard potentials of chlorophyll and iron was calculated from Eqn. 5.

$$E_0^{\mathrm{Chl}} - E_0^{\mathrm{Fe}} = -0.059 \log K \tag{5}$$

TABLE I

CALCULATION OF EQUILIBRIUM CONSTANTS FOR OXIDATION OF CHLOROPHYLL *a* BY $FeCl_3$, IN THE ABSENCE OF DMP

Solution contained about 0.1 μmole chlorophyll in 4 ml, except as noted.

Solvent	*Initial* $FeCl_3$ (μ*moles*)	$\frac{[Chl^+]}{[Chl]}$	K*	$E_0^{Chl} - E_0^{Fe}$ (*mV*)	E_0^{Chl} (*V*) ($E_0^{Fe} = 0.77$ *V*)
Methanol	0.020**	0.01	—	—	
	0.051	0.12	0.19	—	
	0.102	0.78	1.53	−11	
	0.102	0.77	1.43	−4	
	0.153	1.90	2.74	−26	+0.75 ± 0.02
	0.082	3.5***	5.3	−43	
	0.204	4.8	5.45	−43	
	0.307	10.1	6.3	−47	
	0.511	14.3	4.6	−39	
Ethanol (95%)	0.50	0.26	0.014	+109	
	1.00	0.54	0.027	92	
	1.00	0.60	0.035	86	+0.865 ± 0.01
	2.00	0.79	0.025	95	
	10.0	1.69	0.030	90	
Isopropanol	1.00	0.013	0.00023	+210	+0.98

* $K = [Chl^+][Fe^{2+}]/[Chl][Fe^{3+}]$.

** This amount of Fe^{3+} consumed by impurities; correction applied to subsequent data.

*** 0.035 μmole chlorophyll initially.

Ethanol. Oxidized chlorophyll reverts to chlorophyll in less than a minute by a first-order reaction, with at least 75 % recovery if the amount of $FeCl_3$ added is small. The recovered chlorophyll is slowly converted to pheophytin. If the amount of $FeCl_3$ added is large, or if DMP is initially present, the absorption band of the recovered pigment is at 672 nm. In the presence of DMP, Fe^{3+} is reduced to $Fe(DMP)_3^{2+}$ by ethanol within a minute, and the rapid recovery of chlorophyll probably reflects a similarly rapid catalyzed reduction of Fe^{3+} by the solvent. In spite of the instability of the system, values of K in Table I are rather consistent.

Isopropanol. Addition of $FeCl_3$ causes only a slight reduction in chlorophyll absorbance, followed by partial recovery as Fe^{3+} is slowly reduced by the solvent. When DMP is present beforehand, the initial absorbance decrease is much larger. After about 3 min, chlorophyll is entirely converted to a derivative with an absorption band at 671 nm, and 2–3 moles of $Fe(DMP)_3^{2+}$ are formed per mole of chlorophyll.

If one is dealing with the equilibrium

$$\mathrm{Chl} + \mathrm{Fe}^{3+} + 3\,\mathrm{DMP} \rightleftharpoons \mathrm{Chl}^{+} + \mathrm{Fe\,(DMP)_3}^{2+} \tag{6}$$

the standard potential difference can be calculated from the quantity K',

$$K' = \frac{[\mathrm{Chl}^{+}]\,[\mathrm{Fe\,(DMP)_3}^{2+}]}{[\mathrm{Chl}]\,[\mathrm{Fe}^{3+}]} \tag{7}$$

by the expression

$$E_0{}^{\mathrm{Chl}} - E_0{}^{\mathrm{Fe}} = -\,0.059 \log K'\,K_3/[\mathrm{DMP}]^3 \tag{8}$$

in which

$$K_3 = [\mathrm{Fe}^{2+}]\,[\mathrm{DMP}]^3/[\mathrm{Fe(DMP)_3}^{2+}] \tag{9}$$

According to LEE, KOLTHOFF AND LEUSSING[9], K_3 for the ferrous phenanthroline complex is $5 \cdot 10^{-22}$ in water. If this value is even approximately correct for the ferrous DMP complex in isopropanol, the values of K' (Table II), with 10^{-3} M DMP, give $E_0{}^{\mathrm{Chl}} - E_0{}^{\mathrm{Fe}} = +0.8$ V, which seems excessively large.

TABLE II

CALCULATION OF EQUILIBRIUM CONSTANTS FOR OXIDATION OF CHLOROPHYLL *a* BY $FeCl_3$ IN THE PRESENCE OF DMP

Solution contained about 0.1 µmole of chlorophyll and 4.5 µmoles DMP initially, in 4 ml.

Solvent	*Initial* $FeCl_3$ (µ*moles*)	$[Chl^+]/[Chl]$	K'*	K''**	$E_0{}^{Chl} - E_0{}^{Fe}$ (*V*)	$E_0{}^{Chl}$ (*V*)
Isopropanol	0.50	0.32	0.029	0.015	0.28	$+1.05 \pm 0.01$
	1.00	0.54	0.035	0.018	0.28	
	1.00	0.59	0.038	0.020	0.28	
	2.00	0.91	0.036	0.021	0.28	
	2.00	0.62	0.024	0.011	0.29	
	2.00	0.60	0.025	0.011	0.29	
tert.-Butanol	2.00	0.02	—	—	—	

* $K' = [\mathrm{Chl}^+]\,[\mathrm{Fe(DMP)_3}^{2+}]/[\mathrm{Chl}]\,[\mathrm{Fe}^{3+}]$.
** $K'' = [\mathrm{Chl}^+]^2/[\mathrm{Chl}]\,[\mathrm{Fe}^{3+}]$.

However, $Fe(phen)_3^{2+}$ does not form from Fe^{2+} and phenanthroline in one step, but by a relatively slow reaction from the rapidly established equilibrium 10 (ref. 10)

$$Fe^{2+} + phen \rightleftharpoons Fe(phen)^{2+} \quad (10)$$

If the corresponding equilibrium with DMP controls the initial concentration of Chl^+, the standard potential should be calculated from the equation

$$E_0^{Chl} - E_0^{Fe} = -0.059 \log K'' K_1/[DMP] \quad (11)$$

in which

$$K'' = [Chl^+][Fe(DMP)^{2+}]/[Chl][Fe^{3+}] \quad (12)$$

$$K_1 = [Fe^{2+}][DMP]/[Fe(DMP)^{2+}] \quad (13)$$

In Table II, K'' is calculated under the assumption that $[Chl^+] = [Fe(DMP)^{2+}]$. If the value $K_1 = 1.3 \cdot 10^{-6}$ for $Fe(phen)^{2+}$ in water[11] is adopted for $Fe(DMP)^{2+}$ in isopropanol, the values of $E_0^{Chl} - E_0^{Fe}$ listed in Table II are obtained.

tert.-Butanol. Without DMP, there was no detectable reduction of chlorophyll absorbance in the presence of 1 μmole $FeCl_3$. With DMP, about 2 % absorbance loss was noted, but the gradual formation of a greenish precipitate (not chlorophyll) discouraged further work with this system.

DISCUSSION

Values of E_0^{Chl} in methanol, ethanol, and isopropanol have been calculated, relative to E_0^{Fe} in the same solvent, and found to differ considerably. If it is assumed that E_0^{Fe}, relative to the normal hydrogen electrode, is the same in the alcohols as it is in water, +0.77 V, then the values of E_0^{Chl} shown in the last columns of Tables I and II are obtained. These differences might reside in the potential of the Fe^{2+}/Fe^{3+} couple, or of the Chl/Chl^+ couple, or of both. Although the potential of the Fe^{2+}/Fe^{3+} couple might be lower in alcohols than in water, there is no obvious reason why it should vary much from one alcohol to another. Furthermore, results in the presence of DMP are at least qualitatively in agreement with results in its absence (the potential of DMP complexes of Fe would be relatively insensitive to solvent). It is therefore worthwhile to enquire whether there might exist a mechanism for selective stabilization of Chl^+ by the lower alcohols.

One such mechanism would be the reversible addition of alcohol to the 9-carbonyl group of Chl^+, or to the extended system of conjugated double bonds, perhaps as expressed by the reactions

$$Chl^+ + ROH \rightleftharpoons Chl\underset{H}{O^+}R \rightleftharpoons ChlOR + H^+ \quad (14)$$

The species ChlOR would then be a free radical.

On steric grounds, the propensity for addition should decrease in the order methanol > ethanol > isopropanol > *tert.*-butanol, the same order that has been found for addition of alcohols to butyraldehyde to form hemiacetals[12], and for addition to cyclohexanones to form hemiketals[13,14]. In particular, this would account for the special stabilization of Chl^+ in methanol.

Our results indicate that, without stabilization, the standard potential of the

Chl/Chl$^+$ couple would be at least +1.0 V. This value, however, is somewhat uncertain because of our ignorance of the effect of alcohols on the potential of the Fe^{2+}/Fe^{3+} couple. STANIENDA[15,16] has found two reversible, one-electron waves at +0.52 V and +0.77 V (S.C.E.) in the polarographic oxidation of chlorophyll *a* in propionitrile. Although we observed no oxidation of chlorophyll by $FeCl_3$ in acetonitrile, comparison with these results is difficult because of unknown junction potentials and a possibly large differential effect of solvent on the potential of the Fe^{2+}/Fe^{3+} couple.

The ultimate product of oxidation of chlorophyll by excess $FeCl_3$ or by $FeCl_3$ in the presence of DMP, and characterized by a band at 670–672 nm, is not identifiable as a known allomerization product of chlorophyll, such as 10-hydroxy- (or 10-alkoxy-) chlorophyll[17] or a magnesium purpurin 7-lactone ether[18]. However, the 6–7-nm shift of the absorption band to the red, and slight (5–10 %) reduction in intensity, are reminiscent of the effects of chlorine substitution at the δ-position of chlorin e_6 trimethyl ester[19,20]. Our present conditions seem appropriate for this kind of substitution[19].

If a high value is admitted for the potential of oxidation of chlorophyll to unstabilized Chl$^+$, there are consequences of importance to an understanding of photosynthesis. The interpretation of the bleaching of P700 in System I by ferricyanide as an oxidation of chlorophyll[4] becomes intelligible only if the oxidized chlorophyll is stabilized by the attack of some nucleophilic agent. This agent is not likely to be water, because water is much less prone to add to ketones than methanol or ethanol[13]. With regard to System II, a potential of about +1.0 V for unstabilized Chl$^+$ would supply ample energy to drive the oxidation of water to O_2 (+0.815 V at pH 7).

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