Dye-Sensitized Photooxidation of Tocopherols. Correlation between Singlet Oxygen Reactivity and Vitamin E Activity*

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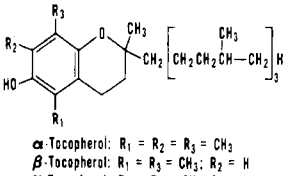
ABSTRACT: The singlet oxygen reactivity of α-, β-, γ-, and δ-tocopherol was determined in methanol with methylene blue as the photosensitizer. The disappearance of tocopherol was followed colorimetrically according to the Emmerie–Engel method. Of the four tocopherols, α was the most reactive ($\beta = 1.4 \times 10^{-4}$ M) and δ was the least ($\beta = 13.5 \times 10^{-4}$ M). α-Tocopherol is one of the most reactive compounds toward singlet oxygen reported in the literature. The reactivity of each tocopherol (α, β, γ, and δ = 1, 0.50, 0.26, and 0.10) correlates well with its vitamin E activity.

Peroxidation *via* a singlet oxygen mechanism has been proposed as the initial step in autoxidation of unsaturated lipids (Rawls and van Santen, 1970; Khan, 1971). Foote *et al.* (1970a,b) recently showed that β-carotene is an extremely good quencher of singlet oxygen and in this role may protect unsaturated lipids from the effects of photodynamic action. Previously, we found that α-tocopherol photooxidized in the presence of a dye sensitizer yielded as major products 4a,5-epoxy-8a-methoxy-α-tocopherol and α-tocoquinone 2,3-oxide (Grams *et al.*, 1972). Further investigation in our laboratory with chemically generated singlet oxygen has shown that these compounds characterize the reaction of α-tocopherol with singlet oxygen (G. W. Grams, 1972). We wanted to determine whether reactivity of members of the tocopherol family toward singlet oxygen could be correlated with their vitamin E activity.



α-Tocopherol: $R_1 = R_2 = R_3 = CH_3$
β-Tocopherol: $R_1 = R_3 = CH_3$; $R_2 = H$
γ-Tocopherol: $R_2 = R_3 = CH_3$; $R_1 = H$
δ-Tocopherol: $R_1 = R_2 = H$; $R_3 = CH_3$

Experimental Section

Materials

d-α-, *d*-β-, *d*-γ, and *d*-δ-Tocopherol were purchased from Eastman Organic Chemicals[1] and were used as received after a purity check by thin-layer chromatography. Methanol from Matheson Coleman, & Bell was spectroquality. Methylene blue (basic blue 9, Matheson, Coleman, & Bell) was dissolved in Spectroquality methanol, diluted to a concentration of 4×10^{-4} M, and kept in the dark until needed. Ethanol was prepared according to the Analytical Methods Committee, Society for Analytical Chemistry, London (1959).

Methods

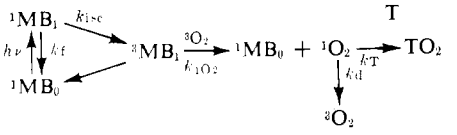
A photolysis apparatus was constructed of plexiglass with five 10-ml Pyrex tubes sealed into a water reservoir. The temperature was maintained at 25° with a Hooke water-circulating pump. The light source was a GE 150-W reflector-flood lamp attached to a variable transformer (Matheson Scientific Co.) for controlling the light intensity. Constant line voltage was maintained with a Sola Electric Co. constant-voltage transformer.

To each of four tubes in the photoreactor was added an identical portion of a standardized tocopherol solution in methanol, 400 μl of the dye solution, and the amount of methanol required to bring the reaction volume to 2.00 ml. Oxygen gas was saturated with methanol in the central tube of the photolysis apparatus and passed in series through the four sample tubes. In this way each methanol solution was saturated with oxygen. After photolysis for a convenient time (α and β, 3 min; γ and δ, 5 min), the reaction mixture from the last tube in the series was removed and diluted with ethanol to a convenient volume for tocopherol analysis by the Emmerie–Engel method (Analytical Methods Committee, 1959). Photolysis was continued and the procedure was repeated after each successive photolysis time interval. A blank containing no tocopherol and a sample not photolyzed were also analyzed with each series of photolyzed samples.

Results and Discussion

Reactivity (β) of the tocopherols was determined in methanol by a modification of the method of Higgins *et al.* (1968). A reaction scheme for dye-sensitized photooxidation (Scheme I) was outlined by Young *et al.* (1970), where MB = methylene blue and T = tocopherol. If $\beta = k_d/k_T$, then $v = -dc/dt = k_{1O_2}[[T]/([T] + \beta)]$ where β is equivalent to the concentration of tocopherol at which half of the singlet oxygen is converted to product. When $[T] \gg \beta$, zero-order kinetics should be observed. For tocopherols in the concentration range of 5×10^{-4} to 20×10^{-4} M, zero-order kinetics was approximated

SCHEME I



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[1] The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

TABLE I: Photooxidation of Tocopherols in Methanol at 25°.

Tocopherol Compound	$c_0 \times 10^4$ (M)	$v_0 \times 10^4$ (M min^{-1})	$1/c_0 \times 10^{-4}$ (M^{-1})	$1/v_0 \times 10^{-4}$ (M^{-1} min)	Equation	$\beta \times 10^4$ (M)
α	3.42	0.183	0.292	5.5	$1/v_0 = 5.27/c_0 \times (3.86 \times 10^4)$	1.4 (±0.4)
	6.86	0.234	0.146	4.3		
	10.33	0.224	0.097	4.5		
	20.64	0.236	0.048	4.2		
				rel std dev = 8%		
β	5.11	0.101	0.196	9.9	$1/v_0 = 18.06/c_0 + (6.34 \times 10^4)$	2.8 (±0.2)
	7.57	0.116	0.132	8.6		
	10.05	0.121	0.100	8.3		
	20.08	0.139	0.050	7.2		
				rel std dev = 13%		
γ	4.94	0.092	0.202	10.9	$1/v_0 = 28.19/c_0 + (5.20 \times 10^4)$	5.4 (±0.3)
	7.36	0.110	0.136	9.1		
	9.95	0.127	0.101	7.9		
	19.66	0.149	0.051	6.7		
				rel std dev = 4%		
δ	5.42	0.038	0.185	26.3	$1/v_0 = 101.11/c_0 + (7.50 \times 10^4)$	13.5 (±0.4)
	7.80	0.049	0.128	20.4		
	10.70	0.060	0.093	16.7		
	22.31	0.082	0.045	12.2		
				rel std dev = 8%		

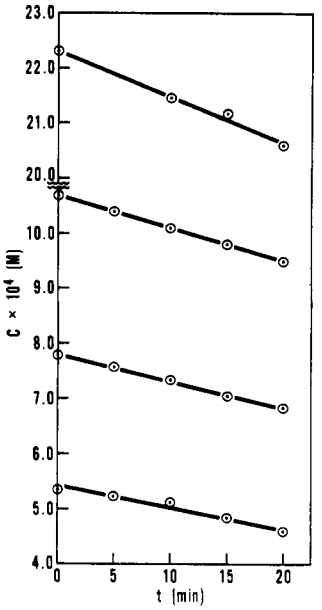

FIGURE 1: Photooxidation of δ-tocopherol.

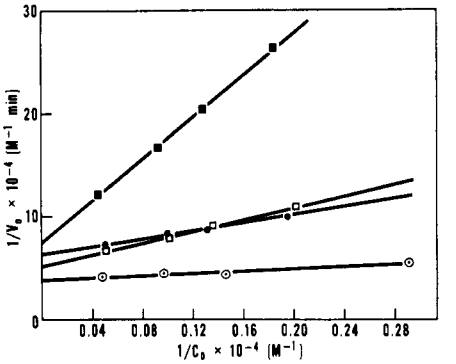

FIGURE 2: Photooxidation of tocopherols. α, ○; β, ●; γ, □; δ, ■.

when conversions were low (<25%). The initial reaction velocity, v_0, at different initial tocopherol concentrations, c_0, was determined by following the disappearance of tocopherol colorimetrically according to the Emmerie–Engel method (Analytical Methods Committee, 1959). The results for δ-tocopherol are shown in Figure 1. Data for all four are summarized in Table I.

At $t = 0$, $v_0 = -dc_0/dt = k_{1O_2} \times c_0/(c_0 + \beta)$ and $1/v_0 = \beta/c_0k_{1O_2} + 1/k_{1O_2}$. Plotting $1/v_0$ *vs.* $1/c_0$ should give a straight line whose intercept is $1/k_{1O_2}$ and slope is β/k_{1O_2}. From Figure 2 the value of β can be calculated as the ratio of the slope to the intercept of that line. The value of β for each tocopherol is given in Table I. α-Tocopherol was the most reactive ($\beta = 1.4 \times 10^{-4}$ M)[2] while δ was the least ($\beta = 13.5 \times 10^{-4}$ M). The reactivity of each tocopherol relative to the α isomer can be calculated from $k_T/k_\alpha = (k_d/k_\alpha) \times (k_T/k_d) = \beta_\alpha/\beta_T$. The value of k_d was estimated to be 1×10^5 sec^{-1} (Higgins *et al.*, 1968). With k_d established, then the rate constant k_α can be calculated: $k_\alpha = k_d/\beta = 1 \times 10^5$ sec^{-1}/1.4×10^{-4} M $= 7 \times 10^8$ M^{-1} sec^{-1}. This rate constant approaches that for a diffusion controlled reaction, 10^{10} M^{-1} sec^{-1}.

Reactivities of tocopherols toward singlet oxygen correlate well with their biological activity (see Table II). When singlet

[2] At the suggestion of a reviewer we repeated the β value determination for α-tocopherol, replacing pure oxygen with air. Since the same β value was obtained ($\beta \times 10^4 = 1.5 \pm 0.2$ M), the reaction of α-tocopherol with dye triplet does not lead to product formation. On this time scale the reaction of α-tocopherol with singlet oxygen appears to be the only observed reaction.

TABLE II: Reactivities of Tocopherols: Correlation between Singlet Oxygen Reactivity and Biological Activity.

Tocopherol Compound	$\beta \times 10^4$	k_T/k_α[b] × 100 (%)	Biological Activity[a]: Respiratory Decline in Rat Livers, *In Vitro* (%)	Respiratory Decline in Rat Livers, *In Vivo* (%)	Erythrocyte Hemolysis, *In Vitro* (%)	Erythrocyte Hemolysis, *In Vivo* (%)
α	1.4	100	100	100	100	100
β	2.8	50	46	55	40	23
γ	5.4	26	26	5	30	3–17
δ	13.5	10	18	4	20	2

[a] Century and Horwitt (1965). [b] Rel std dev = 30%.

oxygen reactivity of each tocopherol is correlated with respiratory decline in rat livers, the reactivity of each tocopherol falls between *in vivo* and *in vitro* biopotency. The correlation between singlet oxygen reactivity and erythrocyte hemolysis is similar except for β-tocopherol, for which the reactivity falls slightly above the range of reported biopotency.

Conclusion

We have demonstrated that oxidation of tocopherols with singlet oxygen in methanol is a good model reaction for certain functions of vitamin E. This correlation suggests that one function of vitamin E may be to protect membranes and lipids from the damaging effects of "active" oxygen.

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Biosynthesis of 5,6-Dimethylbenzimidazole from [1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine*

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ABSTRACT: [1′-^{14}C,5-^{15}N]6,7-Dimethyl-8-ribityllumazine was prepared and added to anaerobically grown cultures of *Propionibacterium shermanii*. After 5 additional days of aerobic growth, the cells were harvested and the vitamin B_{12} was isolated and purified. This biosynthetic B_{12} was hydrolyzed and the resulting 5,6-dimethylbenzimidazole isolated. The ^{15}N and ^{14}C contents of the 5,6-dimethylbenzimidazole were determined and compared with the ^{15}N and ^{14}C contents of the added 6,7-dimethyl-8-ribityllumazine precursor. The location of the ^{14}C within the 5,6-dimethylbenzimidazole was also determined by degradation. The experimental results established that [1′-^{14}C,5-^{15}N]6,7-dimethyl-8-ribityllumazine is an efficient precursor of isotopically labeled 5,6-dimethylbenzimidazole and that the C-1′ carbon atom of 6,7-dimethyl-8-ribityllumazine is incorporated exclusively into the C-2 position of the 5,6-dimethylbenzimidazole. The results also indicated that the C-1′ and the N-5 atoms of 6,7-dimethyl-8-ribityllumazine are incorporated into 5,6-dimethylbenzimidazole as a unit. These results, in conjunction with previous observations, establish that all of the atoms of the 5,6-dimethylbenzimidazole moiety of vitamin B_{12} may be biosynthetically derived from 6,7-dimethyl-8-ribityllumazine.

Recently it was demonstrated that the methyl-^{14}C carbons of 6,7-[^{14}C]dimethyl-8-ribityllumazine are specific precursors of carbon atoms C-4(7) and C-8(9) of DBI[1] in *Propionibacterium shermanii* (Alworth *et al.*, 1971) (*cf.* Figure 1). These results established that the 4,5-dimethyl-1,2-phenylene structural unit of DBI is derived by the same type of bimolecular 6,7-dimethyl-8-ribityllumazine condensation that is involved in the biosynthesis of ring A of riboflavin (Harvey and Plaut, 1966) (*cf.* Figure 1). It had previously been found that the C-2 carbon atom of the DBI moiety of vitamin B_{12} may be derived from the C-1 position of ribose (Alworth *et al.*, 1969). It was proposed, therefore, that all of the carbon atoms of DBI were derived from 6,7-dimethyl-8-ribityllumazine. The

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[1] Abbreviation used is: DBI, 5,6-dimethylbenzimidazole.