REACTIVITY OF SINGLET MOLECULAR OXYGEN WITH CHOLESTEROL IN A PHOSPHOLIPID MEMBRANE MATRIX. A MODEL FOR OXIDATIVE DAMAGE OF MEMBRANES.*

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Summary: Photooxidation of cholesterol in liposomes with hematoporphyrin sensitization has been studied. With liposomal samples in which the hematoporphyrin is incorporated in the membrane, the yield of the characteristic singlet oxygen product, 3β-hydroxycholest-6-ene 5α-hydroperoxide, was approximately 6 times greater than that observed in the samples in which the hematoporphyrin was outside the membrane. Small amounts of 3β-hydroxycholest-5-ene 7α- and 7β-hydroperoxides, radical autoxidation products, were formed in both samples. Photolysis of a dispersion of cholesterol in an aqueous solution of hematoporphyrin gave no singlet oxygen products. It is concluded from these results that endogenous singlet oxygen when formed in the phospholipid membrane has a sufficiently long lifetime to effect oxygenation of cholesterol; whereas exogenous singlet oxygen generated outside the membrane is quenched by solvent before appreciable diffusion into the membrane can occur.

Introduction

Singlet molecular oxygen ($^1O_2$) is receiving increasing attention as a reactive species in various chemical (1, 2) and biological (3 - 7) oxidation reactions. The involvement of singlet oxygen in the photosensitized inactivation of various biologically important substrates and in the photooxidative damage of membranes (8 - 11) has been considered. However, questions concerning the possible importance of $^1O_2$ in biological systems have been raised because of the extremely short lifetime of $^1O_2$ in water (2 μs) (12). As the lifetime of $^1O_2$ in non-aqueous media is significantly longer, singlet oxygen generated in lipophilic membranes should exhibit enhanced reactivity toward substrates incorporated in the membranes. As the lipid matrix differs markedly from a solution, several factors such as the spatial arrangement of the sensitizer and the $^1O_2$ substrate and the rate of diffusion of $^1O_2$ in the lipid matrix will also have to be considered. We have therefore investigated the

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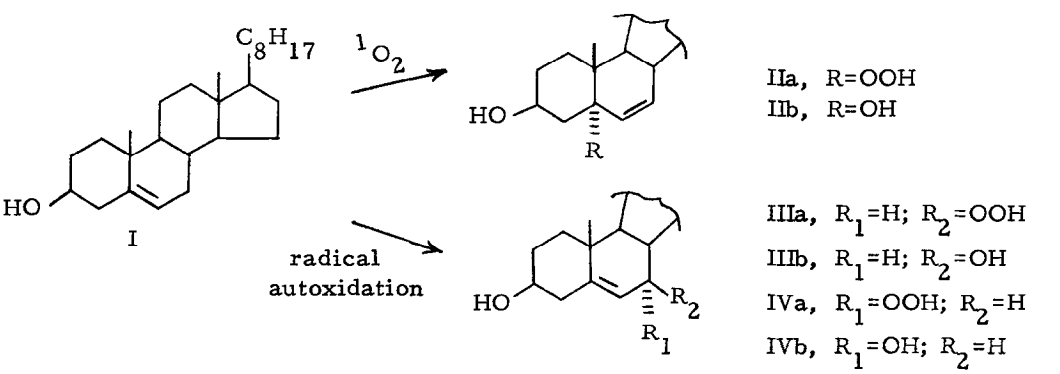
reaction of $^1O_2$ with cholesterol in a phospholipid matrix. Cholesterol was chosen as the substrate for this study for two reasons: not only is cholesterol an important component of biological membranes, but it is also a substrate which yields characteristic singlet oxygen and radical autoxidation products (13, 14). The reaction of $^1O_2$ with cholesterol yields principally the 5α-hydroperoxide IIa via the "ene" reaction while radical autoxidation gives the epimeric 7α and 7β-hydroperoxides, IVa and IIIa, respectively.



IIa, R=OOH
IIb, R=OH

IIIa, R_1=H; R_2=OOH
IIIb, R_1=H; R_2=OH
IVa, R_1=OOH; R_2=H
IVb, R_1=OH; R_2=H

Materials and Methods

Synthetic dipalmitoyl lecithin, cholesterol (recrystallized) and hematoporphyrin were purchased from Sigma. [4-^{14}C] cholesterol (20 μCi/ml) was obtained from Amersham Searle. Silica gel H and precoated Q1 thin layer chromatography plates were purchased from Merck and Quantum Ind., respectively. 3β-hydroxycholest-6-ene 5α-hydroperoxide (IIa), 3β-hydroxycholest-5-ene 7α-hydroperoxide (IVa) and cholest-5-ene-7-one-3β-ol were synthesized (13, 15, 16). Cholest-6-ene-3β, 5α-diol (IIb) and cholest-5-ene-3β, 7α-diol (IVb) were obtained by reduction of the corresponding hydroperoxides (13). A mixture of epimeric IIIb and IVb was prepared by reduction of 7-ketocholesterol with $NaBH_4$.

Liposomal sample 1: A chloroform-methanol (2:1 v/v) solution containing dipalmitoyl lecithin (13.0 μmoles) and [4-^{14}C] cholesterol (0.48 μCi) was added to a 50 ml round-bottomed flask and the organic solvent removed by evaporation under reduced pressure. To the dried lipid film, 10 ml of 0.145 M K-phosphate buffer (pH 7.4) was added and the lipids were thoroughly agitated with a Vortex mixer until the lipid film was no longer detectable on the sides of the flask (17). Liposomal sample 2: Hematoporphyrin in 0.145 M phosphate buffer (pH 7.4) was added to a liposomal sample prepared as above. The concentration of hematoporphyrin was comparable to that in sample 3. Liposomal sample 3: Hematoporphyrin (0.5 mg) was added to the chloroform-methanol solution containing the same amount of dipalmitoyl lecithin and non-labeled and labeled cholesterol. This sample was then treated as in sample 1. The liposomal preparation was dialyzed at ambient temperature against 1 l of a solution containing 0.075 M KCl and 0.075 M NaCl (isotonic solution) for 15 to 18 hr, in order to remove hematoporphyrin that had not been incor-

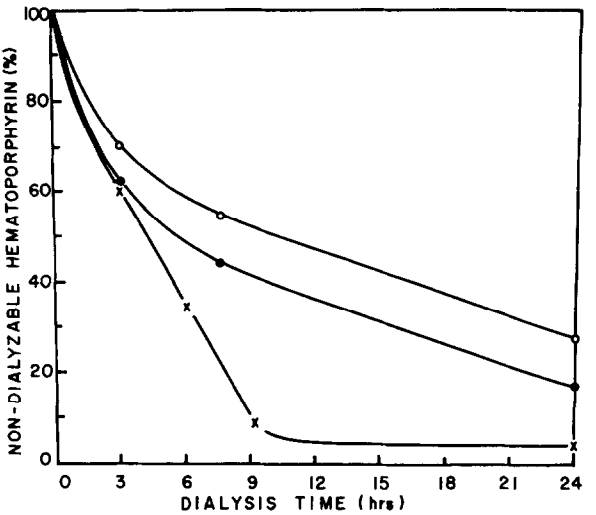


Figure 1. Effect of dialysis on the hematoporphyrin concentration in the liposomal preparations. Ten ml of a free solution of hematoporphyrin and of liposomal samples 2 and 3 were each dialyzed against 1 l of isotonic solution; at the times indicated, a 0.5 ml aliquot of the sample was taken for assay of the hematoporphyrin concentration. The retention of the sensitizer in the samples is shown in Figure 1 as a percentage of initial concentration. The concentration of hematoporphyrin in the liposomal samples was determined by extracting an aliquot of the samples with methanol-chloroform (9:1 v/v). The absorbance at 498 nm was measured and the concentration calculated using an extinction coefficient of 1.12×10^4 M^{-1} cm^{-1}. x—x, buffer solution of hematoporphyrin; ●—●, sample 2; o—o, sample 3.

porated into the liposomes. Liposomal sample 4: Cholesterol (6.5 μmoles) with [4-^{14}C] cholesterol was dissolved in 1.0 ml of acetone; 9.0 ml of 0.145 M K-phosphate buffer (pH 7.4) was added to the acetone solution to make a cholesterol dispersion. Appropriate amounts of hematoporphyrin were added to the dispersed sample.

The irradiations were carried out for 20 min at ambient temperature with a Sylvania 500-W tungsten-halogen lamp. Samples were contained in pyrex vessels and placed 15 cm from the light source. Oxygen was bubbled through the samples during the irradiations. A soft glass or Corning UV-cutoff filter was placed between the light source and the samples.

Results and Discussion

Location of hematoporphyrin: Liposomal samples containing hematoporphyrin were subjected to dialysis against the isotonic solution to determine the extent of hematoporphyrin incorporation into the lipid-membrane space (Fig. 1). Free hematoporphyrin was easily dialyzed through the dialysis tube.

TABLE I.

Formation of Cholesterol Hydroperoxides in Photooxygenated Samples

Sample	Hematoporphyrin (nmoles/ml)	Cholesterol hydroperoxide formation (%)	Cholesterol retained (%)
1	0	2.0	98.0
	0[a]	1.1	98.9
2	14.1	3.9	96.1
	17.1	6.5	93.5
	24.1	8.6	91.4
	18.0[a]	3.6	96.4
	26.6[a]	5.2	94.8
3	13.8	24.6	75.4
	16.8	19.0	81.0
	18.0	28.7	71.3
	26.8[a]	22.9	77.1
	31.2[a]	23.3	76.7
4	26.6[a]	2.9	97.1

The procedure for preparing samples 1, 2, 3 and 4 was given in Methods. Each sample consisted of dipalmitoyl lecithin (13.0 μmoles), cholesterol (6.5 μmoles), [4-^{14}C]cholesterol (0.48 μCi) and various amounts of hematoporphyrin. Each liposomal sample was irradiated by a tungsten-halogen lamp for 20 min at room temperature. Following the irradiations, the reaction mixture was repeatedly extracted with chloroform-methanol solution (2:1 v/v). The pooled extract was evaporated to dryness in vacuo and the resulting residue was dissolved in 0.5 ml of the chloroform-methanol solution. An aliquot (0.05 ml) of this solution was applied to a thin layer plate (silica gel H, 0.25 mm) and developed in ether-cyclohexane (9:1 v/v) under saturated air for 60 min at room temperature. Visualization of the products on the tlc plate was performed by spraying 50% H_2SO_4 and heating. Cholesterol hydroperoxides were determined by spraying N, N, N', N'-tetramethyl-p-phenylenediamine. The yield of cholesterol hydroperoxides is expressed as a percentage of the total radioactivity recovered in each segment of the products in the tlc. All values are the mean of duplicate assays and experimental errors are less than 10% of the values in the table. Typical counts for the experiment were approximately 10,000 at 100%.

[a]A UV-cutoff filter was used for these reactions; a soft glass filter was used for the other experiments.

More than 90 percent of free hematoporphyrin was dialyzable within 9 hrs at 22° C. When hematoporphyrin was added to the liposomal preparation (liposomal sample 2), a considerable amount of hematoporphyrin was retained in the dialysis tube suggesting that hematoporphyrin molecules were adsorbed to the surface of the liposomal membranes. The dialyzing profile of liposomal

TABLE II.

$NaBH_4$ Reduction Products of the Photooxygenated Samples

Sample	1	2	3		4
Hematoporphyrin nmoles/ml	0	26.6	26.8	31.2	26.6
Cholesterol	94.5	90.3	74.8	75.5	92.7
IIb	0.4	2.4	14.0	13.7	0.5
IIIb	1.0	1.5	1.0	1.4	1.4
IVb	1.5	1.5	4.0	3.0	1.5
Unknown Products B	0.6	1.7	4.4	4.2	1.4
Unknown Products A	1.5	1.7	1.5	1.6	2.3
Origin	0.7	0.9	0.5	0.7	0.5

The liposomal preparations (samples 1, 2, 3 and 4) and conditions for the photochemical reaction were the same as those described in the legend for Table I except an increased amount of [4-^{14}C]cholesterol (1.92 μCi). For the reduction of the samples, 1 mg of $NaBH_4$ was added into an aliquot (0.2 ml) of the final extracts (0.5 ml) and the organic solvent was evaporated after reduction. To the residues the same volume of chloroform was added and 0.06 ml of the extracts was placed on tlc plates. The samples were developed under the same solvent system at 4 to 6° C for 120 min. Each product is expressed as a percentage of the total counts recovered from the tlc plate. All values are the mean of duplicate experiments; the errors in experiments are less than 10% of the values reported in the Tables. Total counts recovered from tlc plate were 42502 (cpm) ± 1.7%, 29940 ± 6.6, 24392 ± 6.7, 25893 ± 0.2 and 41203 ± 7.8 in samples 1, 2, 3 and 4, respectively. Reduction of the unreacted cholesterol gave the following analysis: cholesterol (94.9%), cholest-6-ene-3β, 5α-diol(IIb) (0.4%), cholest-5-ene-3β, 7α-diol(IVb) (1.0%), cholest-5-ene-3β, 7β-diol(IIIb) (0.9%), unknown products A (1.6%), unknown products B (0.9%), and origin (0.3%). Total counts recovered was 49700 cpm.

sample 3, in which hematoporphyrin was incorporated into the liposomes, was similar to that of sample 2 except that the amount of non-dialyzed hematoporphyrin of sample 3 was always higher than that of sample 2. The difference between samples 2 and 3 indicates the amount of hematoporphyrin incorporated into liposomes. The value was approximately 10% of the initial concentration of hematoporphyrin. Decreasing the ratio of cholesterol-dipalmitoyl lecithin resulted in less incorporation of hematoporphyrin into the liposomes.

Cholesterol Hydroperoxide Formation: The formation of cholesterol 5α, 7α-, and 7β-hydroperoxides in the various liposomal samples was examined (Table I). A liposomal preparation without sensitizer (sample 1)

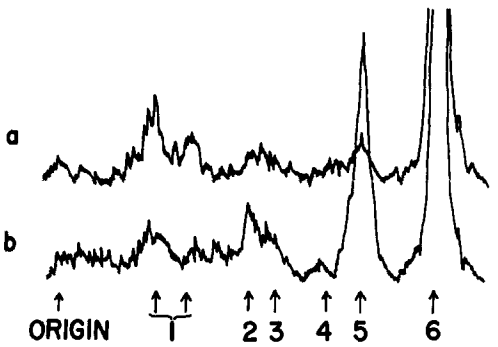


Figure 2. Radioactive scans of thin layer plates of $NaBH_4$ reduction products of samples 2 and 3. Aliquots (80 μl) of the final reduced extracts of samples 2 and 3 were chromatographed on precoated Q1 silica gel plates with ether-cyclohexane (9:1 v/v) at 4-6° C for 120 min. Scan a, sample 2; scan b, sample 3. 1, Unknown products A; 2, unknown products B; 3, cholest-5-ene-3β, 7α-diol(IVb); 4, cholest-5-ene-3β, 7β-diol(IIIb); 5, cholest-6-ene-3β, 5α-diol(IIb); 6, cholesterol (I).

was taken as the control. The yield of hydroperoxides in sample 2 was somewhat dependent on the concentration of hematoporphyrin present in the reaction mixture. However, even at the highest sensitizer concentration the conversion of cholesterol to the hydroperoxides was only 8.6%. These results are in sharp contrast to those obtained with liposomal sample 3 in which 19.0 to 28.7% conversion was obtained. Although the amount of hematoporphyrin incorporated into the membrane may vary with various preparations of sample 3, we consistently observed significant conversion to hydroperoxides. In sample 4, the amount of cholesterol hydroperoxides formed was considerably less than that in samples 2 and 3.

$NaBH_4$ Reduction of the Photooxidized Samples: The primary products of the photooxidations were analyzed as the corresponding diols following $NaBH_4$ reduction (Table II, Fig. 2). The principal product of the photooxidation of sample 3 is the 5α-hydroperoxide IIa formed by the reaction of $^1O_2$ with cholesterol. The radical autoxidation products, 7α- and 7β-hydroperoxides IVa and IIIa, were formed in only small amounts. At the present time we have not attempted to identify the unknown products A and B. Unknown products A were seen in all samples reduced after photoreaction and also observed in a reduced sample of the starting material, cholesterol, in the

same amount. Significant amounts of unknown products B were produced in sample 3. Kulig and Smith (13) have reported that the reaction of singlet oxygen with cholesterol gives in addition to the major product, 5α-hydroperoxide IIa, 6α- and 6β-hydroperoxides as minor products.

The results shown in Table II and Fig. 2 indicate that the photolysis of sample 3 in which the sensitizer was incorporated with the cholesterol in the liposomal membrane gave high conversion of I to IIa with only minor amounts of IIIa and IVa. In contrast, photooxidation of liposomes with adsorbed hematoporphyrin (sample 2) resulted in a much lower yield of IIa. These results are in accord with the expected longer lifetime of $^1O_2$ in the membrane matrix (hydrophobic medium) compared to the lifetime in the aqueous medium outside the membrane. When cholesterol and the sensitizer were separately dispersed in aqueous solution (sample 4), the $^1O_2$ is rapidly quenched with no oxidation of cholesterol. In conclusion, singlet molecular oxygen generated in lipid membrane matrix can oxidize the membrane component, cholesterol, with high efficiency, whereas exogenously generated $^1O_2$ is rapidly quenched by solvent H_2O molecules. This type of reaction may have importance in biological membrane damage.

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