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OXIDATIVE DAMAGE OF RETINAL ROD OUTER SEGMENT MEMBRANES AND THE ROLE OF VITAMIN E

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SUMMARY

Highly purified bovine rod outer segment membranes show loss of structural integrity under an air atmosphere. Obvious ultrastructural changes are preceded by increases in absorbance below 400 nm. These changes are inhibited by Ar or N_2 atmospheres and appear to be due primarily to oxidative damage to the polyunsaturated fatty acids of the membrane lipids. Loss of polyunsaturated fatty acids, formation of malonaldehyde and fluorescent products characteristic of lipid oxidation accompany the spectral alterations. The elevated ultraviolet absorbance can largely be removed from the membranes by gentle extraction of the lipids using phospholipase C and hexane without changing the visible absorbance of rhodopsin.

We have found a large seasonal variation in the endogenous level of α-tocopherol (vitamin E) in the bovine rod outer segment preparations. For much of the year we find that the rod outer segment membranes contain higher levels of α-tocopherol than have been previously reported in biological membranes. Rod outer segments which are low in endogenous tocopherol can be protected from oxygen damage by adding exogenous tocopherol. The rod outer segments are extremely susceptible to oxygen damage due to the unusually high content of polyunsaturated fatty acids in the membrane lipids. The presence of tocopherol inhibits oxygen damage but does not eliminate it. The tocopherol in the rod outer segments is consumed in air, thus complete protection from peroxidation in vitro requires an inert atmosphere as well as high levels of tocopherol.

This work suggests that extensive precautions against oxidative degradation should also be employed in studies of other membrane systems where important deleterious effects of oxygen may be less obvious.

INTRODUCTION

The peroxidizability of unsaturated fatty acids is directly proportional to the number of double bonds [1, 2]. The rod outer segment membranes are unusually rich in polyunsaturated fatty acids. Nearly half of the fatty acids contain six double bonds [3, 4]. Therefore, it is expected that the rod outer segment membranes would

be inherently susceptible to lipid oxidation. There have been many studies of peroxidative damage to polyunsaturated fatty acids in model systems [5–8] and in membranes [9–11]. Membrane structural integrity is lost upon peroxidation [8, 9, 12, 13] and inhibition of membrane function has been reported [14]. Peroxidative damage is not limited to polyunsaturated fatty acids, but can also lead to destruction of amino acids [15, 16], inactivation of enzymes [9, 15] and depolymerization of mucopolysaccharides [17].

Products of polyunsaturated fatty acid peroxidation include conjugated dienes and trienes [5, 18], peroxides [19, 20], malondialdehyde [21] and fluorescent substances [22, 23]. Intact polyunsaturated fatty acids have absorption maxima near 200 nm while the conjugated dienes and trienes absorb maximally near 230 and 270 nm, respectively [5]. Peroxidative damage has been monitored by increases in the ultraviolet absorbance of dienes and malondialdehyde [24], the production of thiobarbituric acid-reactive material [25], the development of fluorescent products [26] and oxygen consumption [27, 28].

The action of α-tocopherol (vitamin E) as a membrane antioxidant and its dietary requirement have been well documented [25, 29–31]. Dilley and McConnell [32] have shown that the bovine rod outer segment is rich in vitamin E. Over the past several years we have observed considerable seasonal variability in many of the properties of purified bovine rod outer segments. In the course of trying to improve the reproducibility and stability of the membranes we have identified lipid peroxidation as a major source of variability of the membrane preparations. We have also explored the effects of variations of the endogenous level of vitamin E and have developed methods to curtail peroxidative damage to the rod outer segments.

MATERIALS AND METHODS

Rod outer segment membrane purification procedure. Membranes were purified from homogenates of bovine retinas according to the method of Raubach et al. [33]. When precautions against oxidation were included in the preparation procedure, the modifications were as follows: buffer solutions were boiled to reduce gas solubility and bubbled with N_2 or Ar during cooling on ice. The retinas were dissected directly into 38% sucrose (w/w) in phosphate buffer previously chilled on ice (0.5 ml/retina). The retinas were either used immediately for the preparation or were stored frozen. If they were to be stored, the retinas were covered with a blanket of argon, covered with Parafilm and a black plastic cap, wrapped in aluminum foil and stored at —20 °C. The purified membranes were stored at 2–6 mg rhodopsin/ml under an Ar atmosphere in a screw top polycarbonate tube covered with Parafilm and a plastic cap and stored on ice. The buffer used throughout these experiments was 68 mM sodium phosphate (pH 7) unless otherwise specified.

Variables that have been normalized to rhodopsin content include regenerable opsin unless otherwise indicated. The content of regenerable opsin was determined by addition of excess 11-*cis*-retinal in the dark [33]. Rod outer segment membranes were diluted with nine volumes of 3% Ammonyx LO (Onyx Chemical; Jersey City, N.J.), phosphate buffer, 10 mM NH_2OH prior to measuring spectra.

Tocopherol assays. The tocopherol content of the membranes was determined quantitatively by two methods. The first we refer to as the rapid determination method

which is a modification of the Hashim and Schuttringer [34] version of the Emmerie-Engle procedure. Membranes containing approx. 100 nmol of rhodopsin (assuming a molar extinction coefficient $\varepsilon_{498\ nm} = 4 \cdot 10^4$) were used. Extracts were manipulated under dim red light during and subsequent to the addition of $FeCl_3$ and α,α-dipyridyl. Absorbance measurements were carried out within 5 min of initiating the reaction. The second assay was according to the method of Dilley and McConnell [32] using membranes containing about 200 nmol of rhodopsin.

In initial experiments a qualitative tocopherol assay was used. A chloroform methanol (1 : 1, v/v) extract of membranes which contained about 23 nmol rhodopsin was spotted on a silica gel GF thin-layer chromatography plate and eluted with chloroform. A minimum of about 0.7 nmol tocopherol could be visualized as a pink spot [35].

Addition of exogenous tocopherol. [^{3}H]Tocopherol (Amersham Searle) was added to a 0.06 M solution of (±)-α-tocopherol (Sigma) in ethanol (Gold Shield) to a final specific activity of 0.11 Ci/mol. An aliquot equivalent to 1 nmol α-tocopherol per nmol of rhodopsin was slowly added to a suspension of purified rod outer segments while stirring. The ethanol concentration in the membrane suspension did not exceed 0.15 %. The suspension was stirred for 10 min under argon and was layered over 30 ml of 26 % of sucrose (w/w) in a 40 ml cellulose nitrate tube and centrifuged at 26 000 rev./min in a Beckman SW 27 rotor for 45 min to pellet the membranes. The supernatant was carefully removed, the membranes were resuspended in buffer and centrifuged through sucrose a second time. Tocopherol incorporation was measured by scintillation counting and by chemical analysis. Counting efficiencies were determined using internal standards.

Oxygen challenging. Oxygen challenging and analysis of malondialdehyde using thiobarbituric acid were modifications of the methods of Dillard and Tappel [26]. The rod outer segment suspensions contained about 4.5 mg rhodopsin/ml in phosphate buffer. Test tubes (12 ml) containing 2–3 ml of the rod outer segment suspensions were taped to Vortex mixers and slowly shaken in the dark while open to the air at room temperature (about 25 °C). Malondialdehyde concentrations are reported as absorbance at 532 nm per mg rhodopsin.

Fatty acid analysis. The method of Morrison and Smith [36] was used. A 6 ft glass column of 10 % SP-2330 on 100/120 Supelcoport (Supelco) was operated at 200 °C in an all glass Perkin-Elmer 3920 gas chromatograph with flame ionization detector.

Phospholipase C treatment and lipid extraction. Purified rod outer segment samples were buffer washed once, water washed once and lyophilized. The lyophilized membranes were stored at −20 °C under an air atmosphere. Membranes were resuspended at 1 mg rhodopsin/ml in phosphate buffer and treated with 0.2 mg/ml phospholipase C (*Clostridium welchii*, Sigma) at 37 °C. Samples were withdrawn at indicated times and spectra measured on aliquots to determine the stability of the 500 nm absorption. The remainder of each sample was water washed three times by centrifugation at 13 000 rev./min in a Sorvall HB-4 rotor, resuspended in water, and aliquots removed for spectra and phosphate analysis. The remainder of each sample was lyophilized and extracted three times in the dark with redistilled hexane using a teflon ball homogenizer. The hexane suspension was centrifuged after each extraction and the final pellet dried in vacuo. The pellet was resuspended in water in a conical

centrifuge tube by inserting a stainless steel spatula into the tube and vortexing. Spectra and phosphate were measured on aliquots of the final suspension in Ammonyx LO as in the other experiments. Phosphate analysis was carried out on each fraction after wet ashing in $HClO_4$ and HNO_3 according to the method of Chen et al. [37].

Electron microscopy. Aliquots of membrane suspensions in phosphate buffer were fixed in 2 % glutaraldehyde for 2 h, washed twice with phosphate buffer and post-fixed in 1 % OsO_4 for 2 h. The membranes were embedded in agar, washed five times with phosphate buffer, washed five times with glass distilled water, dehydrated in graded ethanol and embedded in Spurr's epoxy. Silver-grey sections were cut with glass knives, stained with uranyl acetate and lead citrate and photographed with an RCA EMU-3F instrument at 50 kV.

RESULTS

The open circles in Fig. 1 show the 280 nm/Δ500 nm absorbance ratios of rod outer segment membranes that we prepared from April 1973 to January 1974. Several preparations were done each month and each preparation was made by the same standard procedure [33]. A pronounced seasonal variation is apparent from the higher values of the 280 nm/Δ500 nm absorbance ratio that were obtained for the preparations made from July 1973 to November 1973 as compared to those values obtained during the rest of the year.

The Δ500 nm is the change in absorbance observed upon exposure of a sample to a strong bleaching light and is a measure of the rhodopsin content. The 280 nm absorbance is a sum of absorbance contributions from rhodopsin and opsin as well as impurities and lipid oxidation products (as will be shown later). The contribution of any regenerable opsin to the 280 nm/Δ500 nm ratio can be determined by incubation of the membrane with 11-*cis*-retinal in the dark and measurement of the resulting increase in Δ500 nm. The solid circles in Fig. 1 show the regenerated 280 nm/Δ500 nm ratios. These were found to be significantly higher in the summer, $P \leq 0.01$ % for the July 1973–September 1973 group relative to the April 1973–June 1973, November

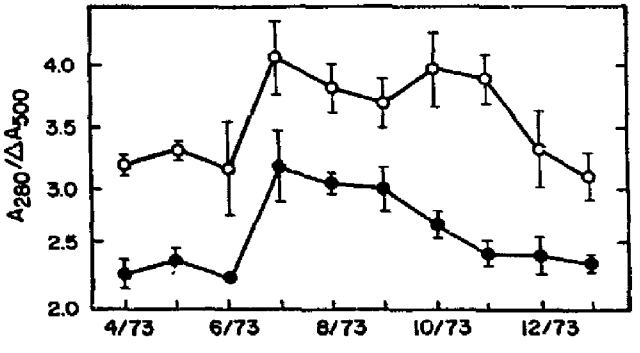

Fig. 1. Monthly average of the 280 nm/Δ 500 nm absorbance ratios of bovine rod outer segment preparations. ΔA_{500} denotes the difference in absorbance between the bleached and unbleached preparations at 500 nm. The open circles are the absorbance ratios prior to addition of 11-*cis*-retinal and the solid circles are the ratios after the regeneration of opsin with excess 11-*cis*-retinal. No precautions against oxidation were taken in these experiments. The error bars represent $\pm$S.D. from the mean.

TABLE I

THE EFFECT OF LIPID DEPLETION ON 280 nm/Δ 500 nm ABSORBANCE RATIOS OF ROD OUTER SEGMENT MEMBRANES

The effects of phospholipase C and phospholipase C combined with hexane extraction on the 280 nm/Δ 500 nm absorbance ratio, and the mol ratio of phosphorus to rhodopsin in rod outer segment membranes. The values are listed according to the length of incubation with phospholipase C. The Δ 500 nm stability exceeded 95 % after enzyme digestion and 90 % after hexane extraction in all cases. A second 0.2 mg/ml enzyme addition was made after the 2 h sample was removed.

Time (h)	Phospholipase C		Phospholipase C+hexane	
	280 nm/Δ 500 nm	Phosphorus/ rhodopsin	280 nm/Δ 500 nm	Phosphorus/ rhodopsin
0	15.2	90	15.0	82
1	12.6	43	8.9	40
2	12.8	33	5.2	30
6	9.3	12.5	4.6	11

1973–January 1974 group. Additional protein impurity bands were not detected in preparations of rod outer segment membranes made from the summer retinas using polyacrylamide gel electrophoresis in sodium dodecyl sulfate. Although sodium dodecyl sulfate solutions of rod outer segment membranes were incubated in 1 % β-mercaptoethanol to break disulfide bonds, more of the opsin did appear in dimer, trimer and higher aggregates in preparations with high ultraviolet/visible absorbance ratios (Nemes, P. P. and Dratz, E., unpublished).

Experiments on lipid depletion from rod outer segment membranes showed that much of the 280 nm absorbing material could be removed from preparations that exhibited high 280 nm/Δ500 nm ratios without decreasing the rhodopsin content. Table I shows the results of such an experiment. The membranes initially had an exceedingly high ultraviolet absorbance (280 nm/Δ500 nm ≈ 15), which could be reduced significantly (280 nm/Δ500 nm = 4.6) by removal of 85 % of the lipids. Unoxidized lipids have no absorbance in the 250–280 nm region, while oxidized polyunsaturated fatty acids do [5, 18]. This suggests that the high values of ultraviolet absorbances were due to lipid degradation products.

The 280 nm/Δ500 nm ratio was found to increase with time in any preparation, if the membranes were exposed to an air atmosphere. Fig. 2 shows a series of absorption spectra as a function of time in air. The spectra show no loss of 500 nm absorbance but show significant absorbance increase throughout the ultraviolet region. The β band near 340 nm becomes less distinct with air exposure because the minima near 300 and 365 nm are filled in. In control samples in which oxygen was carefully excluded by argon or nitrogen bubbling of the buffer and maintenance of an Ar atmosphere, there is essentially no change in the spectrum over the same time period.

These combined results led us to suspect that the poorer spectral ratios found in summer preparations were due to increased lipid oxidation and not due to membrane or protein impurities. We therefore began to use deoxygenated buffers in our preparations and to store membrane samples under Ar or N_2 atmospheres. Since sucrose has been reported to retard lipid oxidation [38], the retinas were dissected

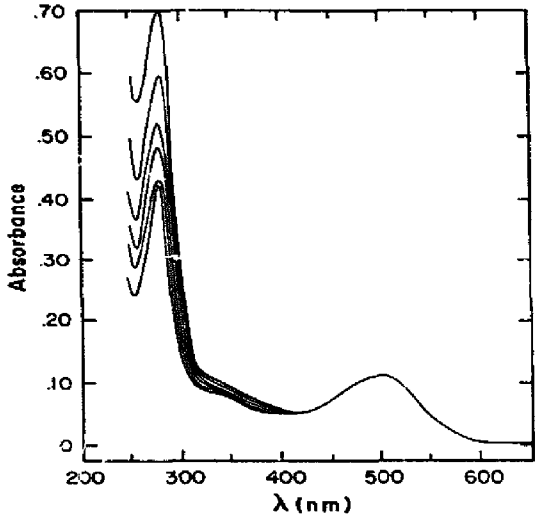

Fig. 2. Absorption spectra of rod outer segment membranes during incubation under an air atmosphere. Membranes were incubated in phosphate buffer at approx. 30 °C. Samples were withdrawn at various times and diluted 9 : 1 with 3 % Ammonyx LO. The spectra shown represent incubation times of 0, 2, 4, 6, 12, and 18 h from the lowest to highest respectively. Rod outer segment membranes (1.3 mg rhodopsin/ml) were prepared from retinas excised in May, 1974.

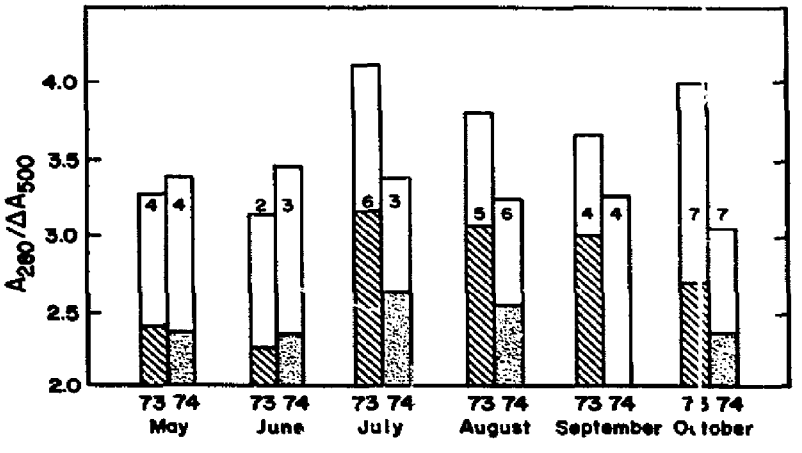

Fig. 3. Comparison of the monthly average of absorbance ratios of bovine rod outer segment preparations for the summer months of 1973 and 1974. During 1973, the preparations were done in air without precautions against oxidation. During 1974 the preparations were done using buffers which had been deoxygenated by bubbling with Ar. Open bars are the ratios prior to the addition of 11-*cis*-retinal and the shaded bars are the ratios after the addition of excess 11-*cis*-retinal. The number above each bar denotes the number of preparations that were done in each month. Regeneration data was not obtained during September, 1974.

into a concentrated sucrose solution. In Fig. 3 the spectral ratios obtained during the late spring and summer of 1974 using the deoxygenated conditions are compared with the ratios of the 1973 preparations done in air. A marked reduction in the unregenerated and regenerated 280 nm/Δ500 nm ratios was found during the summer months in membranes prepared under inert atmospheres. The removal of oxygen is apparently not perfect in these preparations since a slight increase in the spectral ratios of the July 1974 values compared to the May and June 1974 values are observed.

The lipid oxidation reaction is a free radical chain process. The polyunsaturated fatty acids of the membrane lipids initially have all their double bonds unconjugated and isolated by single methylenes. An early stage in the process is double bond migration and the formation of conjugated dienes, which have a peak absorbance near 235 nm and which also absorb strongly at 250 nm [5, 18]. The increase in absorbance at 250 nm will be used to estimate the formation of dienes rather than the absorbance at the 235 nm maximum. The absorbance at 250 nm is near a trough in the spectrum of the rod outer segment membranes and can be measured at the same membrane concentration used for the 280 and 500 nm measurement, whereas the absorbance at 235 nm is too high to measure without an additional dilution. The conjugated dienes are precursors of malondialdehyde [39], as well as of conjugated trienes, which absorb maximally near 270 nm [5].

Fig. 4 shows malondialdehyde concentrations as a function of time plotted together with the 250 nm/280 nm and 280 nm/Δ500 nm spectral ratios of membranes exposed to air at room temperature in the dark. The increase in malondialdehyde level upon air exposure correlates most closely to a concomitant rise in the 250 nm/280 nm spectral ratio. Maintenance of an Ar atmosphere suppresses the spectral changes as shown in Fig. 4 as well as malondialdehyde formation (not shown). Iron has been reported to be a potent catalyst for lipid peroxidation reactions [38, 40]. Fig. 4 shows the accelerating effect of iron addition on the peroxidation of the membranes. The 280 nm/Δ500 nm absorbance ratio initially lags behind that of the 250 nm/280 nm, even after

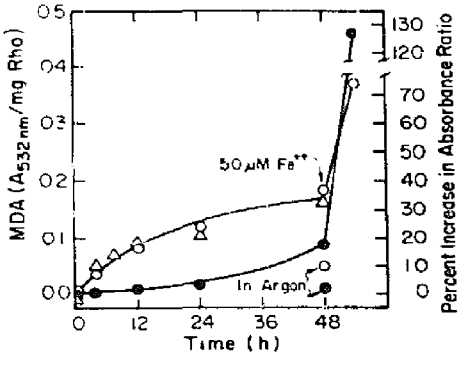

Fig. 4. △—△, malondialdehyde (MDA) concentrations per mg rhodopsin (Rho); ○—○, 250 nm/280 nm absorbance ratios, and ●—●, 280 nm/Δ 500 nm absorbance ratios for rod outer segment membranes as a function of time in air. Samples were incubated at 25 °C in the dark. Absorbance ratios for samples held in Ar for 48 h at 4 °C are also shown. The accelerating effect of 50 μM Fe^{2+} is illustrated. The plus iron data points shown were from a fresh sample that was incubated with iron for 3 h. Rod outer segment membranes (4.5 mg rhodopsin/ml) were obtained from retinas excised during January, 1975.

the addition of iron. The 280 nm/Δ500 nm rises more rapidly at later times. Ethylene diamine tetraacetic acid (EDTA), a potent polyvalent metal ion chelator, has been reported to inhibit non-enzymatic lipid peroxidation in other systems [40, 41]. We have found that the addition of EDTA strongly inhibits the formation of malondialdehyde and the increases in ultraviolet absorbance in rod outer segment membrane suspensions.

The appearance of the rod outer segment membranes in thin sections, as visualized by electron microscopy, is drastically altered by exposure to air, as shown in Fig. 5. The rod outer segment preparation used in this particular experiment was typical of those which are highly sheared during purification, and consists of short stacks of disk membranes (Fig. 5a). Membranes maintained in an Ar atmosphere show no apparent structural degradation (Fig. 5b), while exposure to air for 24 h at room temperature causes substantial destruction of membrane structure (Fig. 5c).

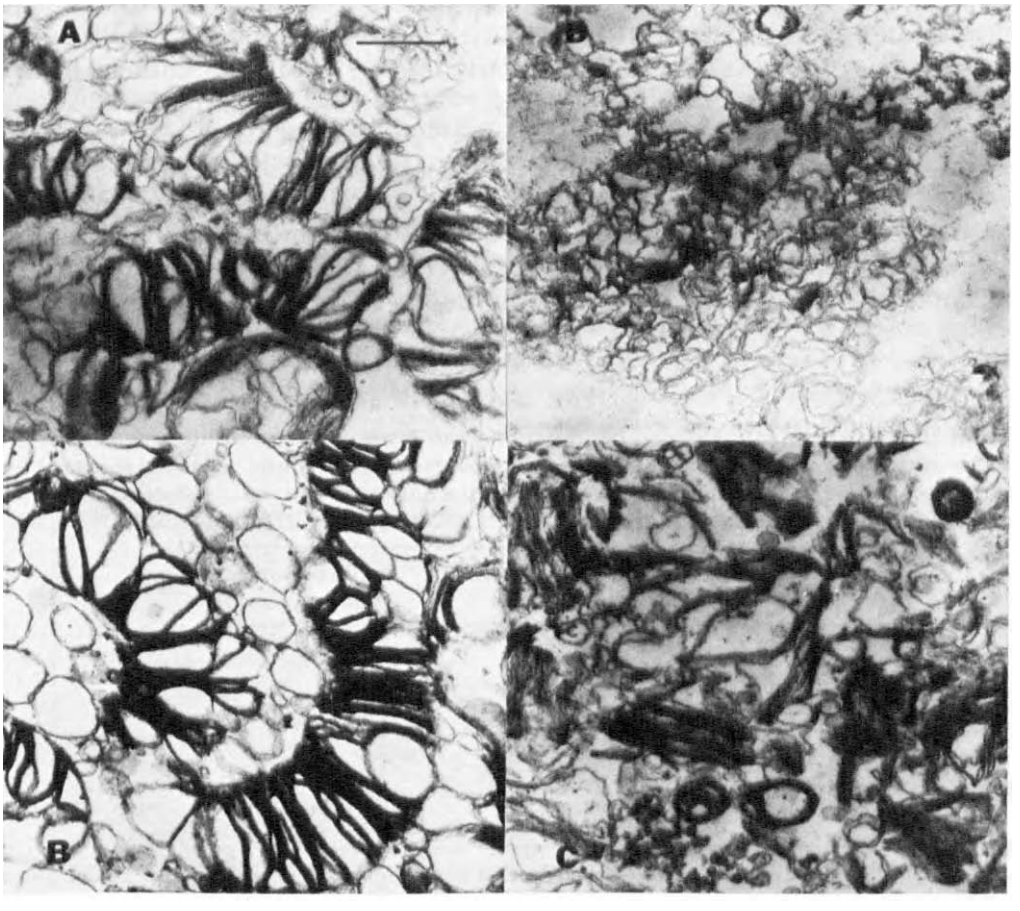

Fig. 5. The effects of oxidation on rod outer segment membrane morphology. Electron micrographs of thin sections of glutaraldehyde-osmium-fixed membrane pellets after exposure to the following conditions: a, zero time sample; b, after 48 h storage at 4 °C under Ar atmosphere; c, after exposure to air for 24 h at 25 °C; and d, after 3 h incubation with 50 μM Fe^{2+} in 140 mM NaCl and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7). The samples are identical to those in Fig. 4. The calibration bar in A is 1 μm.

Iron addition, which produced the large increments of the spectral ratios shown in Fig. 4, resulted in complete disruption of the disk membrane morphology (Fig. 5d) with extensive formation of small vesicles. Membrane samples were withdrawn for fixation from the same material that was used to measure the malondialdehyde and spectral ratios shown in Fig. 4. The extensively oxidized membranes consistently appear to be less osmiophyllic than the control membranes or those maintained under an Ar atmosphere.

The regenerability of rhodopsin is decreased under peroxidizing conditions even though the visible absorption of rhodopsin was not observed to decrease. The regenerability of opsin is reduced from above 90 % in control samples (nitrogen storage, 4 °C for 11 days) to 30 % in peroxidized samples (air storage, 4 °C for 11 days). Bleached membranes stored under nitrogen have the same regenerability as unbleached membranes. Peroxidizing conditions also lead to the loss of free sulfhydryl groups of rhodopsin (Vandenberg, C., Schwartz, S. and Dratz, E., unpublished).

Tocopherol has been reported to be a potent membrane lipid antioxidant [29, 30]. Dilley and McConnell [32] have reported large amounts of tocopherol in rod outer segments. We therefore looked for variations in tocopherol content in the membranes. Preparations made during January 1974 revealed bright pink spots that co-chromatographed with authentic α-tocopherol. In March 1974 we began to use the method of Dilley and McConnell [32] to assay for α-tocopherol and found levels comparable to their results. From June 1974 to October 1974 we were uable to find any tocopherol in the membranes by qualitative or quantitative analysis. The assays used worked well on authentic tocopherol. When tocopherol was absent, the rapid determination spectrophotometric method showed net oxidative equivalents

TABLE II

TOCOPHEROL CONTENT IN ROD OUTER SEGMENT MEMBRANES

Comparison of the endogenous tocopherol per mg rhodopsin from winter rod outer segment preparations, as measured in our laboratory, to the tocopherol levels in rod outer segments reported by Dilley and McConnell [32]. The results of the incorporation of exogenous tocopherol to rod outer segment membranes with undetectable initial endogenous tocopherol levels are included.

	nmol tocopherol/ mg rhodopsin	mol rhodopsin/ mol tocopherol
Endogenous levels		
Present study		
Rapid determination method [a]	1.8	14
Dilley and McConnell method [b]	0.5	50
Published results		
Dilley and McConnell [c]	0.7	36
Exogenous incorporation [d]	1.6	16

[a] Average of 3 November, 1974 preparations by the rapid determination method. Preparation done under Ar atmosphere.

[b] March 1974 preparation assayed by Dilley and McConnell's method, mg rhodopsin does not include regenerable opsin. Preparation done under air atmosphere.

[c] The results have not been corrected for percent tocopherol recovery; mg rhodopsin does not include regenerable opsin.

[d] Incorporation into summer retinas devoid of endogenous tocopherol as assayed by the rapid determination method.

rather than reducing equivalents in the organic extracts. In this test, agents which reduce Fe^{3+} to Fe^{2+} and which are soluble in heptane/ethanol are measured. In some of the heptane/ethanol extracts where no net reducing equivalents are detected it was often observed that the extracts oxidized a trace of Fe^{2+} present in the reagents so that the sample has a lower absorbance than the blank. In November 1974 we again began to detect reducing equivalents in large concentrations in the organic extracts of the membranes. Subsequent tests showed similar high levels of tocopherol throughout the winter months of 1975.

Since membrane preparations with no detectable tocopherol have higher ultraviolet to visible spectral ratios and appear to be undergoing lipid peroxidation, exogenous tocopherol supplementation was used. Table II shows the results of addition of tocopherol to membranes that originally had undetectable levels, together with our analysis of endogenous levels in winter preparations and the values reported by Dilley and McConnell [32]. Nearly equivalent levels of tocopherol incorporation were found both by the rapid determination method for organic-soluble reducing equivalents and by measurement of incorporated tocopherol radioactivity. This indicates that incorporated tocopherol was not significantly oxidized under these conditions. Furthermore, the values obtained by exogenous addition to summer retinas, originally devoid of tocopherol, are very close to the average of three values we obtained for the endogenous level of winter retinas prepared under oxygen-free conditions.

Addition of exogenous tocopherol inhibits lipid oxidation and the increases in ultraviolet absorbance. The extent of inhibition is quite variable. We have not followed the dose response of the inhibition of oxidation by tocopherol in detail. The complex kinetics of lipid oxidation (an autocatalytic free radical chain reaction) implies that the oxidation rate would be expected to be dependent on the oxidative history of the sample (in vivo and in vitro) as well as the tocopherol concentration.

Fig. 6 shows that endogenous tocopherol is consumed in our rod outer segment preparations in air. The rise in the spectral ratios with time, which occurs in the pres-

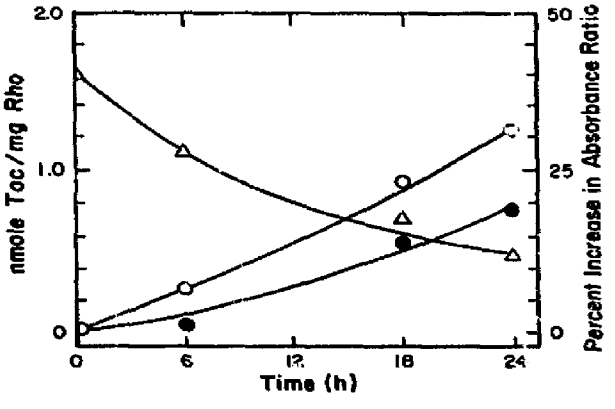

Fig. 6. Endogenous tocopherol (Toc) levels of rod outer segment membranes as a function of time under air atmosphere. Tocopherol levels △—△ are plotted together with the percent increase of the ○—○, 250 nm/280 nm and ●—●, 280 nm/△ 500 nm absorbance ratios of membranes incubated at 25 °C in the dark. Membranes (4.5 mg rhodopsin (Rho)/ml) were obtained from retinas excised during November, 1974.

ence of substantial tocopherol (1.6 nmol tocopherol/mg rhodopsin initially) shows that lipid peroxidation is not completely inhibited by rather high endogenous levels of tocopherol. Numerous other qualitative observations show a much faster lipid oxidation rate at lower tocopherol levels.

Table II shows that the endogenous tocopherol levels reported by Dilley and McConnell [32] are close but somewhat higher than the value we obtained by using their tocopherol analytical procedures under air atmosphere. Dilley and McConnell's [32] preparations were apparently not done under inert atmosphere. The most important difference between their results and ours may well have been the use of retinas obtained from animals with a different tocopherol status.

We have found that peroxidation does not seem to be mediated by bacterial contamination, since it is not inhibited by 0.02 % penicillin (1667 units/mg) and 0.02 % streptomycin. Peroxidation is apparently not mediated by an enzymatic process, since it is not inhibited by boiling the membranes.

The methods used to assay lipid oxidation are convenient and sensitive but indirect. Therefore, direct analysis of the fatty acid composition by gas-liquid chromatography was used as corroboration. Oxidation that produced an absorbance of 5.7/mg protein in the malondialdehyde assay reduced the docosahexaeneoic acid from 42 to 11 % and the total unsaturated fatty acids from 63 to 27 % in the recoverable sample material.

DISCUSSION

We have observed a striking seasonal variation in the apparent purity of bovine rod outer segment membranes prepared in an air atmosphere as evidenced by high ultraviolet to visible absorption ratios. This variation can be largely eliminated by carefully deoxygenating the solutions used in the preparations. These seasonal variations seem to be correlated with the endogenous levels of vitamin E in the rod outer segment membranes. Preparations made in summer, even under inert atmosphere, appear to be devoid of vitamin E. Vitamin E is rapidly consumed under air atmosphere, even in membranes that initially have high levels of endogenous vitamin E. Air atmospheres cause the rapid oxidation of membrane lipids as evidenced by malondialdehyde formation, inhibition of this process by EDTA, acceleration by the addition of iron and loss of polyunsaturated fatty acids.

The oxidized lipids form products which absorb in the ultraviolet. The 280 nm/Δ500 nm and the 250 nm/Δ500 nm absorbance ratios of rod outer segment membrane preparations are elevated as a result of oxidative damage. The absolute values of these ratios depend on the fraction of rhodopsin bleached. The 250 nm/280 nm absorbance ratio which is independent of bleaching is a useful routine measure of the early stages of oxidation, since initially, the 280 nm absorbance rises much more slowly than the 250 nm absorbance. Much of the elevated ultraviolet absorbance can be removed, even in heavily oxidized rod outer segment membranes, by lipid depletion without removal of rhodopsin. Large amounts of lipofuscin-like fluorescent products characteristic of lipid oxidation [26] arise during exposure of rod outer segment membranes to air (Huffaker, T. and Dratz, E., unpublished).

DeGrip et al. [42] also reported that 280 nm/Δ500 nm ratios in bovine rod outer segment preparations were higher in the summer compared to winter prepara-

tions. They were able to account for this elevation by the presence of higher levels of regenerable opsin in their summer preparations. Measurement of and correction for regenerable opsin was not sufficient to account for the elevated 280 nm/Δ500 nm absorbance ratios in our summer preparations made in air. However, we find that during the summer it is possible to maintain ratios which are nearly as low as those in winter preparations by the use of inert atmospheres.

Fresh cow's milk develops an oxidized, rancid flavor during the summer [43] on the west coast of the United States. The oxidized flavor is due to oxidation of the phospholipids of the milk fat globule membranes [44]. The oxidized flavor can be eliminated in the milk by supplementing the animals' diets with tocopherol or by adding exogenous tocopherol to the milk [45]. The cattle on the west coast eat dry feed in the summer which is very low in vitamin E, whereas in winter they eat green feed which is high in the vitamin. The difference between our observations and those of Bonting's group in Holland is presumably due to differences in dietary tocopherol between the cattle in the Netherlands and the cattle in California. Lipid oxidation, if present in their preparations, is apparently not as regular or as severe a phenomenon as in ours. It is clear that increased tendency for membrane lipid oxidation is associated with low levels of antioxidants in animal diets [29, 46]. The problem we have observed with bovine retinas is not expected to be restricted to this material. Lipid oxidation can be a dominant experimental variable in membrane studies if it is not monitored and controlled.

Lipid oxidation in air atmospheres can be quite rapid in vitro even in rod outer segment membranes with high levels of endogenous vitamin E. The vitamin E is consumed in air atmospheres and peroxidation occurs at an easily measurable rate at room temperature. We have not measured the temperature coefficient of the reaction with care. Peroxidation is slowed at 4 °C and accelerated at 37 °C, but the temperature coefficient does not appear to be unusually large. Boiling the preparations does not inhibit peroxidation of the rod outer segment lipids so it is not an enzymatic process. Non-enzymatic lipid peroxidation of membranes is known to be strongly catalyzed by iron impurities [40] and is essentially stopped by the addition of EDTA [40, 41]. EDTA may not be a generally useful membrane antioxidant, because it complexes Ca^{2+} and Mg^{2+} which are probably needed for membrane stability. Phosphate also acts as a weak antioxidant presumably by weakly complexing iron ions [38].

The membrane structure is strikingly damaged by substantial peroxidation as seen by the loss of disk membrane morphology in the electron microscope. Since a functional assay for the rod outer segment disk membrane is not yet available, we cannot assess the importance of inhibiting peroxidation for the maintenance of rod outer segment disk membrane function. Williams et al. [47] have reported that the rate of the metarhodopsin I to metarhodopsin II reaction is affected by exposure to air. We have found that opsin loses its ability to combine with retinal to regenerate rhodopsin with increased lipid peroxidation, but this probably cannot be regarded as a measure of a primary functional lesion in the disk membrane.

Lipid peroxidation has damaging effects on membrane integrity [8, 9, 12, 13] and function [14] in other systems, and inhibits enzyme activity [9, 15]. Peroxidative damage is not limited to membrane lipids, but can also lead to mucopolysaccharide depolymerization [17], and amino acid destruction, especially histidine, tyrosine, lysine [15] and cysteine [16]. It seems plausible that lipid peroxidation is an important

variable in studies of disk membrane function and should be controlled. Most studies of disk membrane function have been carried out on frog preparations made from animals sold by commercial suppliers. The nutritional state and general health of commercial frogs is often very poor [48]. Bownds and co-workers [49] have gone to some length to improve the nutritional state of the frogs they used to study the rod outer segment membranes. It is likely that extensive precautions against lipid peroxidation in vitro is also important to maintain intact membranes. The lack of an assay for disk membrane function in vitro is a major difficulty in the study of the functional mechanisms of rhodopsin and rod outer segments.

The retina must be heavily supplied with oxygen in vivo because of its unusually rapid rate of oxidative metabolism [50]. The electrical response of the photoreceptor is highly susceptible to anoxia [51, 52] and glucose deprivation [53]. Furthermore, the photoreceptors sometimes function in very high light levels, which approach photon fluxes of 10^{10} above threshold sensitivity. Visible light can be a potentiator of oxidation in the presence of suitable pigments [54]. It has been reported that light absorbed by rhodopsin sensitizes lipid oxidation in purified frog rod outer segments [55]. Lipids throughout the turkey eye are oxidized in visible light in vivo and the oxidation is potentiated by dietary iron [56].

Vitamin E appears to be the major first line of defense against lipid peroxidation [57]. Vitamin E is able to retard lipid peroxidation by quenching free radical chain initiators. It seems highly likely, but has not yet been proven that vitamin E is an important antioxidant in rod outer segments in vivo. We have shown that vitamin E is rapidly consumed in rod outer segment preparations in air in vitro. Therefore, it seems probable that there must be other potent protective mechanisms against peroxidation in vivo in addition to vitamin E. We are in the process of studying such mechanisms. Enzyme activities such as superoxide dismutase [59] and glutathione peroxidase [57, 60] have been implicated in the protection against lipid peroxidation of other tissues. Superoxide dismutase has been reported to occur in high concentrations in rod outer segments [61] and catalase has been reported in the pigment epithelium [58]

Retinal photoreceptor membranes are damaged by surprisingly low levels of light in vivo in a variety of experimental animals [62–66]. The photoreceptors in vivo are also highly sensitive to degeneration caused by elevated oxygen levels for brief periods [51, 52, 67]. It may be that the protective mechanisms against lipid oxidation are relatively easy to overload. Some types of retinal degeneration in animals and humans might therefore be expected to be due to deficiencies in the natural protective mechanisms against peroxidative damage. It has recently been reported that vitamin E deficiency leads to the degeneration of the macular region of the retina (the fovea and surrounding area) as seen in two species of monkeys [68].

The deleterious effects of lipid oxidation on membrane structure [8, 9, 28, 38, 69, 70] and function [14] have been reported. However, precautions against lipid oxidation do not seem to be widespread [71] in studies of biological membranes. Lipid peroxidation may be an important uncontrolled variable in many areas of cell biology and membrane biochemistry.

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