*Molecular and Cellular Biochemistry* **197:** 173–178, 1999.


# The effect of α tocopherol, all-trans retinol and retinyl palmitate on the non enzymatic lipid peroxidation of rod outer segments

M. Guajardo,[1] A. Terrasa[1] and A. Catalá[1,2]

[1]*Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata CC 296, La Plata, Argentina;* [2]*Carrera del Investigador Científico, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina*

Received 15 May 1998; accepted 31 August 1998

## Abstract

The effect of α tocopherol, all-trans retinol and retinyl palmitate on the non enzymatic lipid peroxidation induced by ascorbate-$Fe^{2+}$ of rod outer segment membranes isolated from bovine retina was examined. The inhibition of light emission (maximal induced chemiluminescence) by α tocopherol, all-trans retinol and retinyl palmitate was concentration dependent. All trans retinol showed a substantial degree of inhibition against ascorbate-$Fe^{2+}$ induced lipid peroxidation in rod outer segment membranes that was 10 times higher than the observed in the presence of either α tocopherol or retinyl palmitate. Inhibition of lipid peroxidation of rod outer segment membranes by α tocopherol and retinyl palmitate was almost linear for up to 0,5 µmol vitamin/mg membrane protein, whereas all-trans retinol showed linearity up to 0,1 µmol vitamin/mg membrane protein. Incubation of rod outer segments with increasing amounts of low molecular weight cytosolic proteins carrying 1-[$^{14}$C] linoleic acid, [$^{3}$H] retinyl palmitate or [$^{3}$H] all-trans retinol during the lipid peroxidation process produced a net transfer of ligand from soluble protein to membranes. Linoleic acid was 4 times more effectively transferred to rod outer segment membranes than all-trans retinol or retinyl palmitate. Incubation of rod outer segments with delipidated low molecular weight cytosolic proteins produced inhibition of lipid peroxidation. The inhibitory effect was increased when the soluble retinal protein fraction containing α tocopherol was used. These data provide strong support for the role of all-trans retinol as the major retinal antioxidant and open the way for many fruitful studies on the interaction and precise roles of low molecular weight cytosolic retinal proteins involved in the binding of antioxidant hydrophobic compounds with rod outer segments. (Mol Cell Biochem **197**: 173–178, 1999)

*Key words*: lipid-peroxidation, rod outer segments, α tocopherol, all-trans retinol, retinyl palmitate

## Introduction

The antioxidant activity of retinoids and α tocopherol against lipid peroxidation has long been observed both *in vitro* [1, 2] and *in vivo* [3, 4].The preventive effect of natural and synthetic antioxidants on lipid peroxidation has been assayed in bovine retina, purified rod outer segments and retinal pigment epithelium [5]. Studies from Livrea laboratory using crude retinal membranes has demonstrated reciprocal protective effects of all-trans retinol and α tocopherol during lipid peroxidation induced by the water soluble azo initiator 2,2'-azobis (2-amidinopropane) hydrochloride [6]. Previous evidence in our laboratory showed that lipoperoxidation of rod outer segments of bovine retina is inhibited by a fraction containing soluble binding proteins for fatty acids (Terrasa *et al*. [7]). This cytosolic protein is also able to bind vitamin A derivatives such as retinyl palmitate. Since α tocopherol and all-trans retinol also bind to cytosolic proteins, this prompted us to evaluate the effect of α tocopherol, all-trans retinol and retinyl palmitate alone or bound to its intracellular

*Address for offprints*: Angel Catalá, Cátedra de Bioquímica, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata CC 296, 1900 La Plata, Argentina

binding proteins on the non enzymatic lipid peroxidation induced by ascorbate-$Fe^{2+}$ of rod outer segment membranes isolated from bovine retina.

## Materials and methods

Bovine eyes were obtained from Gorina slaughterhouse, Sephadex G75 and Sepharosa 4 B were from Pharmacia Fine Chemicals Inc. Piscataway N. J. BSA (Fraction V) and cytochrome c were obtained from Wako Pure Chemical Industries Ltd. Japan. Lipidex 1000 was purchased from Sigma, USA. Retinol all- trans (20-Methyl-$^3$H ) was purchased from NEN TM Life Science Products Bostons MA USA 73.5 Ci/mmol. Retinol Palmitate, All-trans Type IV: Sigma Grade Synthetic, (±) α Tocopherol Approx 95% and Retinol all-trans Approx 70% Synthetic were from Sigma Chemical Co.[$^3$H] retinyl esters were prepared by incubation of Retinol all-trans (20-Methyl-$^3$H ) with microsomes from bovine retinal pigment epithelium using the methods described by Saari and Bredberg [8].

All other reagents and chemicals were of analytical grade from Sigma Chemical Company.

*Rod outer segment membranes preparation*

Bovine eyes were removed at slaughter, transported in ice to laboratory and retinas taken out within 1–2 h. Retinas were separated and rod outer segments were isolated from the whole retina by continuous sucrose density gradient centrifugation by methods that have been described by Borggreven *et al*. [9]

*Preparation of retinal cytosol*

Fifteen retinas were collected in an ice-cold solution of 0.25 m sucrose, 10 mm Tris-HCl pH 7.4 0.1 mm PMFS (1 ml per retina) and homogenized using a Teflon-glass Potter Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 13500 x g and the resulting supernatant was applied to a Sepharose 4 B column (1.6 x 12 cm) equilibrated and eluted with 10 mM Tris- HCl pH 7.4 , 0.005% $NaN_3$, in order to separate particulate material from cytosol [10]. The soluble fraction appearing in the volume (24–36 ml) was pooled and concentrated using a ultrafiltration cell MFS-UHP43-MW CO 10000 at $pN_2$ = 2–5 kg/cm$^2$. All operatios were performed at 4°C.

*Delipidation of retinal cytosol*

6 ml of retinal cytosol containing 25 mg protein were subjected to chromatography on Lipidex 1000 at 37°C according to the method described by Glatz *et al*. [11]. The column used was ( 8 x 1.2 cm ) equilibrated and eluted with buffer 10 mM phosphate, pH 7.4 containing 0.005\% $NaN_3$.

*Incubation of retinal cytosol with α-tocopherol*

65 nmol of α-tocopherol/mg of cytosolic protein were incubated with 6 ml of delipidated retinal cytosol.

*Isolation of retinal cytosolic protein with hydrophobic ligands properties*

Fractions containing low molecular weight proteins were preparated by filtration of retinal delipidated cytosol and retinal delipidated cytosol (25 mg of each) incubated with α-tocopherol, in a Sephadex G75 column (3 x 40 cm) calibrated with BSA (66 kDa) and cytochrome c ( 12.4 kDa). An eluent 10 mM Tris-HCl pH 7.4, 0.005% $NaN_3$ was used. The flow rate was 90 ml/h. The protein fraction (150–200 ml) were pooled and concentrated by ultrafiltration cell.

*Incubation of all trans [$^3$H] retinol and all trans [$^3$H ] retinyl palmitate with retinal cytosol*

3 µCi of [$^3$H ] all-trans retinol was incubated with 6 ml cytosol (49.84 mg Pt.) and applied on a Sephadex G75 column (3 x 40 cm), flow rate 90 ml/h, equilibrated and eluted with 10 mM Tris-HCl pH 7.4, 0.005% $NaN_3$. Samples of 5 ml fractions were collected and radioactivity determined in each fraction with a liquid Scintillation Counter Packard 1900 TR. Measurements of absorbance at 280 nm were carried out simultaneously. Two large fraction were separated.

One in the high molecular weight range and another enriched with low molecular weight proteins having hidrophobic ligand binding properties. A similar procedure was done by using 4.5 µCi all trans [$^3$ H] retinyl palmitate with 6 ml cytosol (20.76 mg Pt.) Retinal cytosol incubated with [$^3$H]all-trans retinol or its fatty acid ester showed association of radioactivity with a low molecular weight protein.

*Lipid peroxidation of rod outer segment membranes*

Chemiluminescence and lipid peroxidation were initiated by adding ascorbate to rod outer segments [12]. Membranes at a concentration of 0.25 mg of protein were treated previously with different amounts of α-tocopherol, all-trans retinol or retinyl palmitate and then incubated at 37°C with 0.01 M phosphate buffer (pH 7.4), 0.4 mM ascorbate, final volume 2 ml. Phosphate buffer is contaminated with sufficient iron

to provide the necessary ferrous or ferric iron for lipoperoxidation. Membrane preparations which lacked ascorbate were carried out simultaneously. Membrane light emission was determined over a 180 min period, chemiluminescence was recorded as cpm every 10 min and the decrease of the sum of the total chemiluminescence was used to calculate % inhibition.

*Transfer of 1-$^{14}$C linoleic acid, $^{3}$H retinyl ester or $^{3}$H all-trans retinol from low molecular weight cytosolic proteins to rod outer segment membranes*

Rod outer segment membranes (0.25 mg protein) were incubated as indicated before in the presence of low molecular weight proteins (0.2–0.4–0.6 mg) containing either 1-$^{14}$C linoleic acid, $^{3}$H retinyl ester or $^{3}$H all-trans retinol. When the incubation period necessary to obtain total lipid peroxidation was reached, the sample was centrifuged at 13500 x g 10 min in an Eppendorf microcentrifuge. Aliquots of the supernatant and the whole pellet were used to measure radioactivity as previously described (Avanzatti and Catalá, [13]). The amount of fatty acid or retinoid transferred to rod outer segment membranes was calculated from the sum of radioactivity present in pellet and soluble material.

## Results

After incubation of rod outer segments in an ascorbate-$Fe^{2+}$ system, at 37°C during 120 or 180 min, it was observed that the total cpm originated from light emission (chemiluminescence) was lower in those membranes incubated in the presence of the antioxidants analyzed. The times courses of the chemiluminescence resulting from the addition of four different concentrations of either α tocopherol, all- trans retinol and retinyl palmitate to rod outer segment membranes are shown in Fig.1A, B and C respectivelly. After addition of ascorbate (0.4 mM) , rod outer segment membranes showed maximal light emission at approximately 40 min. An additional induction time of 40 min was observed when retinyl palmitate (0.125 µmol mg prot$^{-1}$) was incorporated into the membranes. This induction time was not observed when α tocopherol or all-trans retinol were used as antioxidants. Inhibition of lipid peroxidation by α tocopherol and retinyl palmitate, was almost linear up to 0.5 µmol mg prot$^{-1}$, whereas all-trans retinol inhibition showed linearity up to 0.1 µmol mg prot$^{-1}$ Fig. 2. IC50 values were calculated from the inhibition curve of antioxidants on the chemiluminescence rates (data no showed). All-trans retinol showed a substantial degree of inhibition against ascorbate-$Fe^{2+}$ induced lipid peroxidation in rod outer segment membranes that was 10 times higher than the observed in the presence of either α

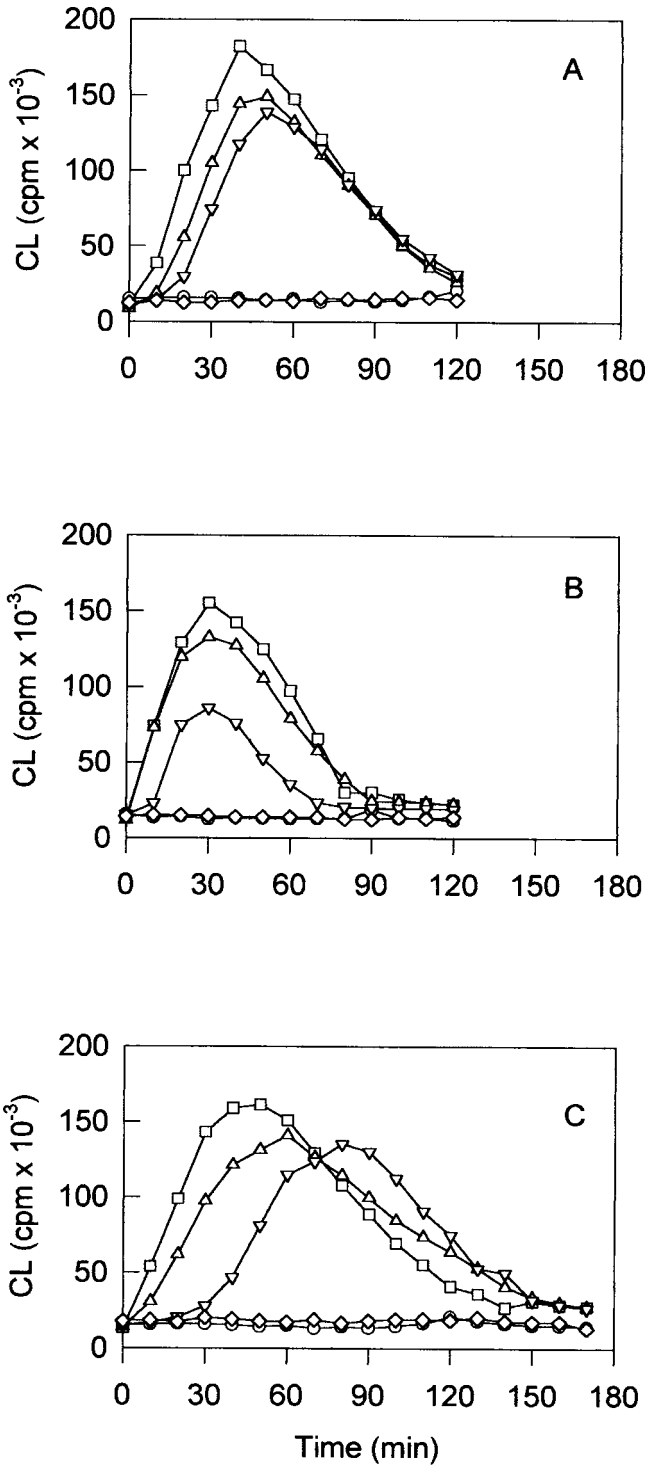


*Fig. 1.* Lipid peroxidation of rod outer segment membranes (0.25 mg of protein) with the addition of (A) 0, □–□; 0.125, Δ–Δ; 0.250, ∇–∇ and ◊–◊, 1 µmol α tocopherol/mg protein rod outer segment membranes. (B) 0, □–□; 0.0125, Δ–Δ; 0.025, ∇–∇ and ◊–◊, 0.1 µmol all-trans retinol/mg protein rod outer segment membranes. (C) 0, □–□; 0.0625, Δ–Δ; 0.125, ∇–∇ and ◊–◊, 0.5 µmol retinyl palmitate/mg protein rod outer segment membranes. In all cases control without ascorbic acid.

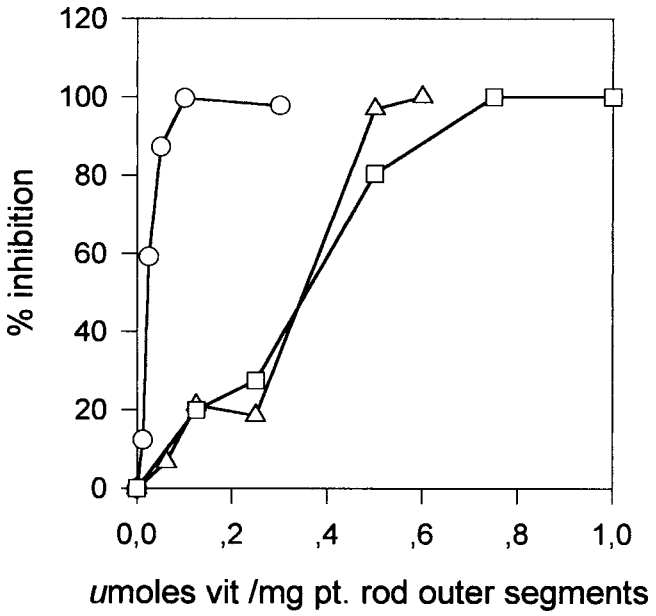


*Fig. 2.* Inhibition of lipid peroxidation of rod outer segment membranes by all-trans retinol ○–○, alpha tocopherol □–□ and retinyl palmitate Δ–Δ.

tocopherol or retinyl palmitate. Figure 3 shows typical gel filtration experiments in which two major fractions with fatty acid or retinoid binding properties are observed when retinal cytosol incubated either with 1-$^{14}$ C linoleic acid, $^{3}$H retinyl palmitate or $^{3}$H all-trans retinol was analyzed. Incubation of rod outer segments with increasing amounts of low molecular weight cytosolic proteins carrying 1-$^{14}$C linoleic acid, $^{3}$H retinyl palmitate or $^{3}$H all-trans retinol during the lipid peroxidation process produced a net transfer of ligand from soluble protein to membranes. Thus when 0.4 and 0.6 mg of cytosolic protein were used linoleic acid was 4 times more effectively transferred to rod outer segment membranes than all-trans retinol or retinyl palmitate which could be an indication of the relative affinities of ligand to cytosolic retinal proteins and/or rod outer segment membranes Fig. 4. A decrease of chemiluminescence was observed with the addition of increasing amount of delipidated or α tocopherol containing fraction isolated from retinal cytosol to rod outer segment membranes. The inhibitory effect was more pronounced when the α tocopherol containing fraction was used. Figure 5.

## Discussion

In a previous study [7] we have demonstrated that after incubation of rod outer segment membranes in an ascorbate-$Fe^{2+}$ system, at 37°C during 120 min, the total cpm originated from light emission (chemiluminescence) was lower in those

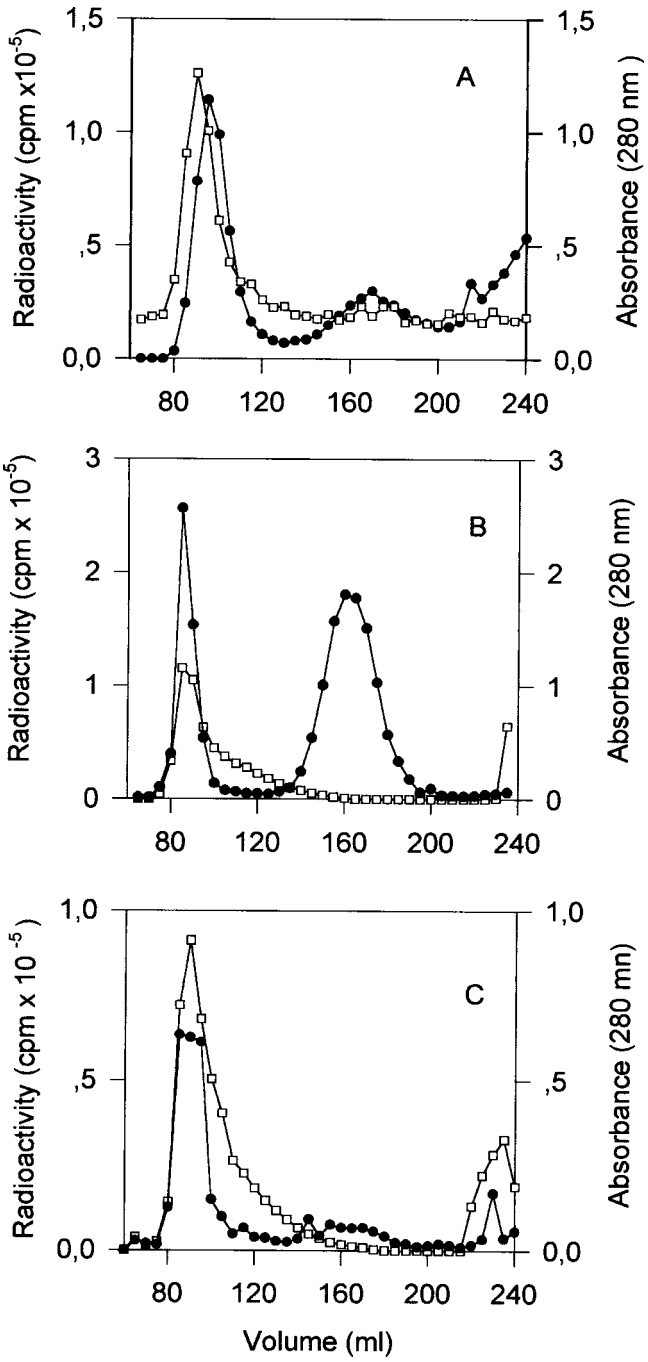


*Fig. 3.* About 1 µCi of [1-$^{14}$C] linoleic acid + retinal cytosol (62,23 mg pt) (A) 3 µCi of [$^{3}$H]all-trans retinol + retinal cytosol (49,84 mg pt) (B) or 4,5 µCi of [$^{3}$H]retinyl ester + retinal cytosol (20,76 mg pt) (C) were chromatographed in Sephadex G75 (3 x 40 cm) , flow rate 90 ml/h, 5 ml fractions, elution buffer 10 mM Tris-HCl pH 7.4. Samples were collected and the radioactivity ●–● and absorbance to 280 nm □–□ was measured.

membranes incubated in the presence of soluble binding proteins for fatty acids. The fatty acid composition of rod outer segment membranes was substantially modified when

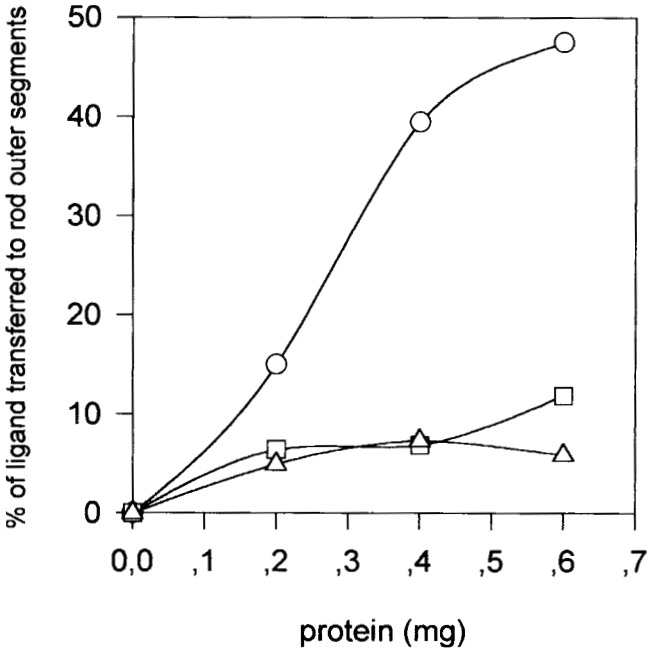


*Fig. 4.* Transfer of [1-$^{14}$C] linoleic acid ○–○, all-trans retinol □–□ or retinyl palmitate Δ–Δ. from low molecular weight proteins of retinal cytosol to rod outer segment membranes.

subjected to non-enzymatic lipoperoxidation with a considerable decrease of docosahexaenoic acid (22:6 n3) and arachidonic acid, (20:4 n6). Since in the range 14–15 kDa coexist cytosolic proteins that bind long chain fatty acids,

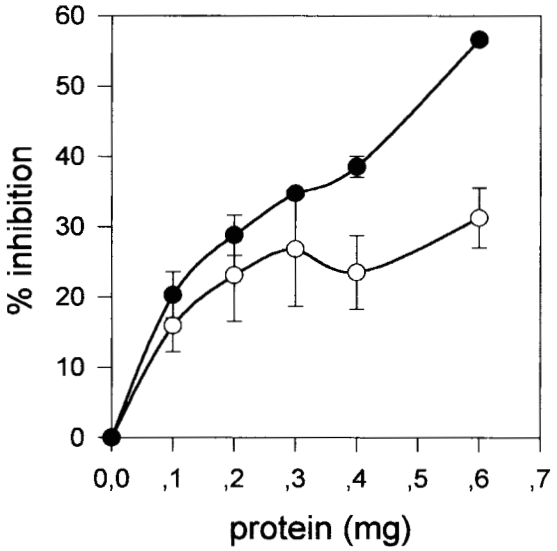


*Fig. 5.* Inhibition of ascorbate-$Fe^{2+}$ induced chemiluminescence of rod outer segment membranes by retinal low molecular weight cytosolic proteins; delipidated ○–○ or loaded with α tocopherol ●–●. Results are mean ± S.D. of three determinations.

retinol palmitate, α tocopherol and all-trans retinol, we decided to analyze the effect of this lipid soluble vitamins alone or bound to its cytosolic proteins on the non enzymatic lipid peroxidation of rod outer segment membranes in an ascorbate-$Fe^{2+}$ system. Lipophylic vitamins such as α tocopherol, retinyl palmitate and all-trans retinol have been well studied in several tissues because of their antioxidant effects, however the mechanisms for their ability to prevent lipid peroxidation process are not fully understood. In accordance with the proposed mechanism by which polyenes such as carotenoids and retinoids may intervene during lipid peroxidation, retinol reacts by lipoperoxyl radical ($LO_2$*) addition to cyclohexenyl ring. The resulting retinoid-adduct carbon-centered radical may add a second lipoperoxyl radical. This route can support effective lipoperoxyl radical scavenging activity of all-trans retinol wich may acts as a chain-breaking antioxidants [14]. We have developed an *in vitro* model membrane system in order to assess the relative effectiveness of three lipophilic antioxidants on the non enzymatic lipid peroxidation of rod outer segment membranes. Our results clearly indicate that the antioxidants studied may act directly when are incorporated into the rod outer segment membrane or when added to our *in vitro* system bound to low molecular weight cytosolic proteins. In this regard it is important to note that the exchange of all-trans retinoids (retinal, retinol, retinyl palmitate) between phosphatidylcholine (PC) vesicles and rod outer segment membranes has been investigated using 11,12 $^3$H labelled compounds. Rod outer segment membranes take up relatively small amounts of retinoids (retinyl palmitate less than retinol less than retinal) and rapidly lose 60–90% of their label in the presence of PC-vesicles. Apparently, rod outer segment membranes have a much lower affinity for retinoids than other artificial or natural membranes investigated so far [15]. 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 [16]. Retinyl palmitate and retinyl stearate are of central importance in the visual cycle because they are the major storage molecules for retinol. In some tissues (e.g. liver) the amount of vitamin A (mostly in the form of retinyl ester) is positively correlated with both the amount of α tocopherol and age [17]. Vitamin E is thought to be important for protection of polyunsaturated fatty acids from oxidative damage. The vitamin-E content in various structures of the retina has been determined, the highest concentration of vitamin was located in the retinal pigment epithelium followed by the outer segments of the photoreceptor cells [18]. It appears, that these lipophilic antioxidants, can be delivered to by its specific cytosolic binding proteins to their action sites on the membrane where they play their antioxidant effect . Taken together our results indicate that several lipophilic compounds including vitamin E, retinyl palmitate and retinol contribute

to the antioxidant protection of the polyunsaturated fatty acids present in rod outer segment membrane phospholipids. To our knowledge, the presence of α-tocopherol binding protein in retinal cytosol, has not been described. Further studies on the interaction of these low molecular weight cytosolic proteins involved in the binding of the lipophilic antioxidants analized in this study may be of importance in the knowledge of retinal function.

## Acknowledgement

This work was carried out with grants from Comisión de Investigaciones Científicas Pcia. Bs. As. (CIC) and Secretaria de Ciencia y Técnica , Universidad Nacional de La Plata. The authors wish to thank to Med Vet Cesar Arcemis for the excellent technical assistance.

## References

1. Das NP: Effects of vitamin A and its analogs on non enzymatic lipid peroxidation in rat brain mitochondria. J Neurochem 52: 585–588, 1989
2. Tesoriere L, Bongiorno A, Pintaudi AM, D'Anna R, D'Arpa D , Livrea MA: Synergistic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes Arch Biochem Biophys 326: 57–63, 1996
3. Zanetti R , Catalá A: Ascorbate-$Fe^{2+}$ lipid-peroxidation of rat liver microsomes: Effect of vitamin E and cytosolic proteins. Mol Cell Biochem (in press)
4. Ross D, Moldeus P: Antioxidant defence systems and oxidative stress. In: C. Vigo-Pelfrey (ed.). Membrane Lipid Oxidation. Boca Raton, Fl: CRC, pp 151–170, 1991
5. Ueda T, Ueda T, Armstrong D: Preventive effect of natural and synthetic antioxidants on lipid peroxidation in the mammalian eye. Ophtalmic Res 28: 184–192, 1996
6. Tesoriere L, Bongiorno A, Re R, Livrea MA: Reciprocal protective effects of all-trans retinol and alpha-tocopherol during lipid peroxidation in retinal membranes.Biochem Mol Biol Int 37: 1–7, 1995
7. Terrasa A, Guajardo M, Catalá A: Lipoperoxidation of rod outer segments of bovine retina is inhibited by soluble binding proteins for fatty acids. Mol Cell Biochem 178: 181–186, 1998
8. Saari JC, Bredberg DL: CoA-and non-CoA-dependent retinol esterification in retinal pigment epithelium. J Biol Chem 263: 8084–8090, 1988
9. Borggreven JMPM, Daemen FJM , Bonting SL: The lipid composition of native and hexane-extracted cattle rod outer segments. Biochim Biophys Acta 202: 374–381, 1970
10. Tangen O, Jonsson J, Orrenius S: Isolation of rat liver microsomes by gel filtration. Analyt Biochem 54: 597–603, 1973
11. Glatz JFC, Veerkamp JH: A radiochemical procedure for the assay of fatty acid binding by proteins. Analyt Biochem 132: 89–95, 1983
12. Wright JR, Rumbaugh RC, Colby HD, Miles PR: The relationship between chemiluminescence and lipid peroxidation in rat hepatic microsomes. Arch Biochem Biophys 192: 344–351, 1979
13. Avanzatti B, Catalá A: Exchange of palmitic acid from cytosolic proteins to microsomes, mitochondria and lipid vesicles Acta Physiol Latinoam 32: 267–276, 1982
14. Tesoriere L, D'Arpa D, Re R, Livrea MA: Antioxidant reactions of all-trans retinol in phospholipid bilayers: Effect of oxygen partial pressure, radical fluxes and retinol concentration. Arch Biochem Biophys 343 No 1: 13–18, 1997
15. Groenendijk GW, De Grip WJ, Daemen FJ: Rod outer segment membranes: good acceptors for retinoids? Vision Res 24: 1623–1627, 1984
16. Farnsworth CC, Dratz EA: Oxidative damage of retinal rod outer segment membranes and the role of vitamin E. Biochim Biophys Acta 443: 556–570, 1976
17. Crabtree DV, Snodderly DM, Adler AJ: Retinyl palmitate in macaque retina-retinal pigment epithelium-choroid: Distribution and correlation with age and vitamin E. Exp Eye Res 64(3): 455–463, 1997
18. Stephens RJ, Negi DS, Short SM, van Kuijk FJ, Dratz EA, Thomas DW: Vitamin E distribution in ocular tissues following long-term dietary depletion and supplementation as determined by micro-dissection and gas chromatography-mass spectrometry. Exp Eye Res 47: 237–245, 1988