POTENTIATION OF INTRAOCULAR ABSORPTION AND DRUG METABOLISM OF *N*-ACETYLCARNOSINE LUBRICANT EYE DROPS: DRUG INTERACTION WITH SIGHT THREATENING LIPID PEROXIDES IN THE TREATMENT FOR AGE-RELATED EYE DISEASES

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SUMMARY

Cataract is the dominant cause of blindness worldwide. Studies of the morphological structure and biophysical changes of the lens in human senile cataracts have demonstrated the disappearance of normal fiber structure in the opaque region of the lens and the disintegration of the lens fiber plasma membrane in the lens tissue. Morphological and biochemical techniques have revealed the regions in human cataractous lenses in which the plasma membrane derangement occurs as the primary light scattering centers which cause the observed lens opacity.

Human cataract formation is mostly considered to be a multi-factorial disease; however, oxidative stress might be one of the leading causes for both nuclear and cortical cataract. Phospholipid molecules modified with oxygen, accumulating in the lipid bilayer, change its geometry and impair lipid-lipid and protein-lipid interactions in lenticular fiber membranes. Electron microscopy data of human lenses

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at various stages of age-related cataract document that these disruptions were globules, vacuoles, multilamellar membranes, and clusters of highly undulating membranes. The opaque shades of cortical cataracts represent cohorts of locally affected fibres segregated from unaffected neighbouring fibres by plasma membranes. Other potential scattering centers found throughout the mature cataract nucleus included variations in staining density between adjacent cells, enlarged extracellular spaces between undulating membrane pairs, and protein-like deposits in the extracellular space. These affected parts had membranes with a fine globular aspect and in cross-section proved to be filled with medium to large globular elements.

Lipid peroxidation (LPO) is a pathogenetic and causative factor of cataract. Increased concentrations of primary molecular LPO products (diene conjugates, lipid hydroperoxides, fatty acid oxy-derivatives) and end fluorescent LPO products were detected in the lipid moieties of the aqueous humor samples and human lenses obtained from patients with senile and complicated cataracts as compared to normal donors.

Utilizing the pharmacokinetic studies and the specific purity *N*-acetylcarnosine (NAC) ingredient as a source of pharmacological principal L-carnosine, we have created an ophthalmic time-release prodrug form combined with a muco-adhesive lubricant compound carboxymethylcellulose and other essential corneal absorption promoter excipients tailoring the increased intraocular absorption of L-carnosine in the aqueous humor and optimizing its specific effect in producing the basic antioxidant activity *in vivo* and reducing toxic effects of lipid peroxides to the crystalline lens. L-Carnosine that finds its way into the aqueous humor can accumulate in the lens tissue for a reasonable period of time. However, administration of pure L-carnosine (1% solution) to the rabbit eye (instillation, subconjunctival injection) does not lead to accumulation of this natural compound in the aqueous humor over 30 min in concentration exceeding that in the placebo-treated matched eyes, and its effective concentration is exhausted more rapidly.

The NAC prodrug eye drops optimize the clinical effects for the treatment of ophthalmic disorders (such as prevention and reversal of cataracts in human and animal [canine] eyes). The data provided predict a particular NAC ophthalmic prodrug's clinical effect; the suitable magnitude and duration of this effect suggest dose-related

bioavailability of L-carnosine released from NAC in the aqueous humor of the anterior eye segment.

The ophthalmic NAC drug shows promise in the treatment of a range of ophthalmic disorders which have a component of oxidative stress in their genesis (including cataract and after-cataract, glaucoma, dry eye, vitreous floaters, inflammatory disorders, corneal, retinal and systemic diseases [such as diabetes mellitus and its ophthalmic complications]). The clinical efficacy of *N*-acetylcarnosine lubricant eye drops in ripe cataracts and retinal disorders can be enhanced in combined treatment with a patented oral formulation (Can-C Plus) of non-hydrolyzed carnosine including synergistic compounds (histidine, D-panthethine) with chaperone activity towards lens crystallins and oral supplementation with *N*-acetylcysteine providing an alternate means of boosting reduced glutathione (GSH) synthesis in the lens.

KEY WORDS

age-related cataracts, electron microscopy, human cataractous lenses, membrane derangement, oxidative stress, lipid peroxides, *N*-acetyl-carnosine, carboxymethylcellulose, ophthalmic prodrug, L-carnosine, pharmacokinetic studies, dipeptides, intraocular absorption, aqueous humor, antioxidant, ophthalmic disorders

"*The eyes have one language everywhere.*"

George Herbert

INTRODUCTION

Cataract is an opacity of the natural, crystalline lens of the eye and remains the most frequent cause of blindness in the world today. The World Health Organization (WHO) estimates that 50% (17 million) of persons currently blind worldwide are blind from cataract /1/ and 28,000 new cases are reported daily worldwide /2/. Because of increasing life expectancy and the resulting expansion of the elderly population, prevalent cases of blinding cataract are expected to double by the year 2010 /1,2/. Current evidence supports the view that cataractogenesis is a multifactorial process, in which combination of

more closely linked events induces subtle post-translational modifications in the lens structural proteins, enhancing their aggregation, fragmentation, and precipitation, resulting eventually in lens opacification /3/.

It is well established that a major factor involved in the development of cataract is oxidative insult /3-15/. Oxidative stress associated with the formation of lipid peroxides is suggested to contribute to pathological processes in ageing and systemic diseases, such as diabetes mellitus, atherosclerosis, chronic renal failure, inflammation and retinal degenerative diseases known as statistically significant risk factors for cataract /16-21/. The observation that lipid peroxides are elevated in the lens membranes of some patients with cataract has drawn attention to these toxic oxidants /22-25/. Lipid peroxides can cause cataract, producing damage to both the cell membrane and cytosol regions /21,24-30/. At the membrane, lipid hydroperoxides induce changes in permeability /31-34/, refashion the microviscosity (order) of its lipid-protein environment /35-38/, cause an uncoupling of the membrane-bound enzyme Na,K-ATPase and oxidative inhibition of Ca^{2+}-ATPase in several tissues including the lens /39-41/. Within the cell, lipid peroxides can damage DNA /42/, induce a drop in total glutathione and dramatic change in the redox ratio of glutathione, and lead to the appearance of new fluorophores and large protein aggregates with low solubility (clouding matrix) in the lens matter /15,42-46/.

The crystalline lens is washed with the aqueous humor which influences the overall viability of the lens organ and its metabolism through the anterior portion of the eye. Both development and cataract disease are affected by the environment of the respective tissues of the eye. The aqueous humor contains about 4 μg/ml high density lipoproteins, which evidently take part in the renewal of lipid compositions of the lens /47,48/. The oxidative modification of lipoproteins in the presence of trace amounts of transition metals (copper or iron) is variously associated with lipid peroxidation (LPO), an increase in net negative charge, hydrolysis of phospholipid and fragmentation of apoprotein B (for review see /49/), and the oxidized lipid moieties of lipoprotein particles can be implicated in the lens toxicity triggering cataractogenesis /50,51/. Since LPO is clinically important in many of the pathological effects and ageing accompanying cataract formation, new therapeutic modalities should treat

the incessant infliction of damage to the lens cells and biomolecules by reactive lipid peroxides and reactive oxygen species in the lack of important metabolic detoxification of these cataractogenic factors.

Previously published data suggest that L-carnosine (β-alanyl-L-histidine) has excellent potential to act as a natural antioxidant with hydroxyl-radical and singlet oxygen-scavenging and lipid peroxidase activities /44,52,53/; thus it may be useful to prevent, or partially reverse, lens cataracts /44,54/. Exogenous carnosine entering the organism intravenously, intraperitoneally, with food, or topically to the eye, is not accumulated by the tissues but is excreted in the urine or destroyed by carnosinase, a dipeptidase present in blood plasma, liver, kidney and other tissues, except muscle, and, probably, lens /44,54-57/.

The *N*-acetyl derivatives of histidine, carnosine and anserine exist in cardiac and skeletal mammalian muscles and the total concentration of these imidazoles may lie within the measured range of that of L-carnosine in skeletal muscle (i.e.~10 mM) /58/. Among 29 dipeptides of the carnosine family tested as potential substrates for a highly purified human serum carnosinase preparation, *N*-acetylcarnosine (NAC) and a few other compounds were not hydrolyzed /56/, thus promising a prolongation of physiological responses to the therapeutic treatments. A knowledge of corneal and iris/ciliary body esterase activity, in particular, acetylesterase (EC 3.1.1.6) and, in addition to esterase, the identified *N*-acetyltransferase activities /59/, prompted the development of a prodrug of L-carnosine in its ophthalmic application as antioxidant such as the chemically characterized *N*-acetylated form of the dipeptide /60,61/. Due to relative hydrophobicity compared with L-carnosine, NAC might penetrate through the cornea gradually, thus maintaining longer the active therapeutic concentration of L-carnosine in the aqueous humor of the treated eye /60,61/. Clinical studies have been validated in several reports, by prospective evaluation of lens opacities and visual function in cataractous patients when the physiologically acceptable solution of NAC was applied topically to the eye /62-64/.

However, the ophthalmic drugs will only produce their desired action if they are present at the site of action in sufficient quantities, and the design of the formulation and choice of the route of administration must take this into account. Topically applied agents should produce effective levels mainly in the anterior segment. Drugs

administered in the form of eye drops have to run a gauntlet of events which will, in the main, reduce the resultant concentration at the effector site. Utilizing the specific purity of the (L)-natural isoform NAC ingredient as a source of pharmacological principal L-carnosine, we have created an ophthalmic formulation, which contains varying amounts of active ingredients, tailoring the enhanced intraocular absorption of the beneficial ingredient, the dipeptide L-carnosine, to optimize its specific effect and purpose in producing the basic antioxidant activity *in vivo* and reducing toxic effects of lipid peroxides to the crystalline lens.

MATERIALS AND METHODS

Patients

Aqueous humor and human lenses were obtained from patients undergoing intracapsular cryoextraction of senile or complicated cataracts and used as the test material. This project was approved by the ethics commission of Helmholtz Research Institute for Eye Diseases (no. 008/136/30). The control aqueous and transparent human lenses were obtained from eyes donated for corneal grafting supplied from a corneal transplant bank within 15 h post mortem. The mean age at surgery and the average donor age was 59 years ranging from 16 to 89 years. Both groups contained an almost equal number of males and females.

Aqueous humor sampling from human eyes

After retrobulbar and lid anaesthesia the two vertical recti muscles were fixed. A stab incision was performed transcorneally 1 mm from the limbus in the temporal superior quadrant. Aqueous humor from human eyes (about 0.1-0.2 ml per eye) was aspirated from the anterior chamber of each eye with a 25-gauge needle connected to a tuberculin syringe immediately before surgery and shipped on wet ice under sterile conditions. Human aqueous humor was then briefly centrifuged at low speed to remove any cellular debris and the aliquots of fresh aqueous humor from each patient were immediately used for lipid extraction.

Lenses

The test and control material consisted of opaque human lenses at different stages of cataract or normal human lenses. Before surgery all the lenses were studied by biomicroscopy and assigned to cataractous or transparent lenses in line with the clinical characteristics of clouding. Transparent lenses were obtained from freshly enucleated eyes of chinchilla rabbits. Normal mouse lenses were extracted from the eyeballs by the posterior approach from mice of the strain C57BL or hybrids F1 (CBA x C57BL) resistant to cataract formation. In all cases the integrity of the lens capsule was preserved. The lenses removed were briefly rinsed in Hanks' medium for 20-30 s and then immediately placed into a moist chamber. They were handled with the greatest care when being placed inside and being removed from their individual containers for measurements. The lenses were either used directly after the extraction procedure or surgery, or at least 1-3 h were allowed to elapse between the dissection from the eye and the start of the measurement. This delay did not influence the results.

The development of quantitative morphometric criteria for evaluation of the lens opacities was presented by us previously /65,66/. All opacities were graded by their area and density, as judged by the degree of clouding (Fig. 1). The integral degree of lens clouding (IDLC) was estimated as the ratio between the opacity zone area corresponding to maximum levels of the lens optical density values and the whole lens surface area expressed as a percentage. This measuring procedure was brief (5-10 min) and did not influence the further lens behaviour.

Electron-microscopic study

For electron microscopy, whole lenses or pieces of lens tissue were fixed with 4% glutaraldehyde prepared in phosphate buffer (0.1 M, pH 7.2-7.4), and then with 1% OsO_4 solution, as described previously /15, 67,68/. Afterwards, the samples were washed with 50% ethyl alcohol. Lens dehydration was performed in alcohol of increasing concentration: 60%, then in 2% uranylacetate on 70%, 80%, 96% and 100% alcohol. Furthermore, the samples were put in absolute acetone and were then kept in a mixture of acetone with epone resin (2:1):(1:2). The lenses kept in epone were maintained at 37°C (24 h) and then at 60°C (48 h). Sections for electron microscopy were prepared

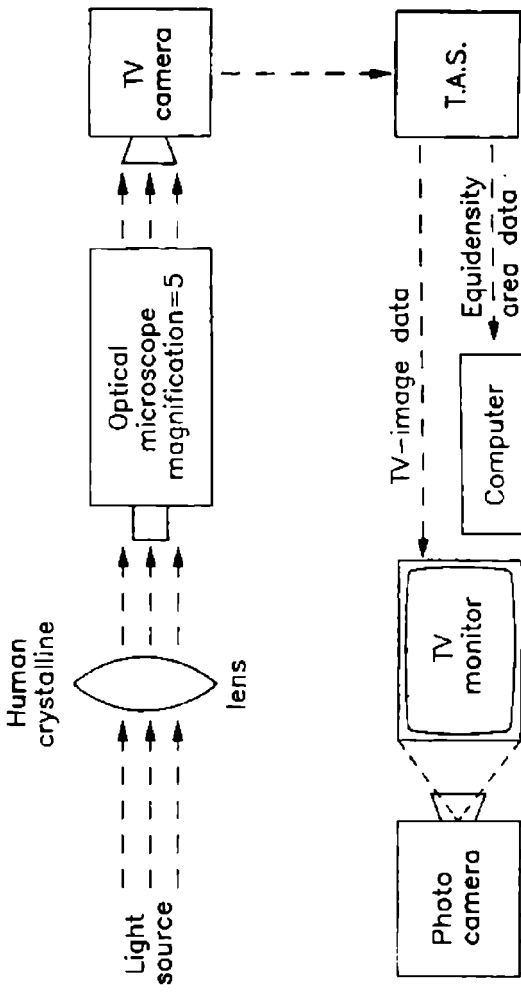


Fig. 1: Image analysis procedure which makes it possible to obtain an image of the lens, to determine values of the optical density in different parts of the lens, to divide the lens surface into zones of defined density range, to measure the areas of equidensities and to evaluate the precise topography of every zone. All parameters are estimated quantitatively by use of a computer. The lens is illuminated in a frontal projection, adjusting the intensity of the illuminating light to make the transmission through each lens constant. An electronically produced measuring field is determined by a circular mask whose position is under the control of the analyser. To control its position, the coordinates of the central point of the mask are automatically digitally defined. The image is then scanned and the density at each coordinate stored in the computer.

perpendicularly to the surface in the medial lens zone area on an LKB-200 11 ultramicrotome. The contrasting stain was performed with lead nitrate by the Reynolds method /69/. The samples were examined on a Jem 100 B electron microscope.

Lipid extraction and quantification in the aqueous humor

Aliquots of fresh aqueous humor from each patient or animal were immediately extracted into 40 volumes of chloroform-methanol (2:1, v/v) with 4-methyl-2,6-ditert-butyl-phenol (BHT) antioxidant addition (0.5 mg/100 ml) during 10 min. The obtained extract was washed according to the modified Folch procedure, and the chloroform layer was recovered and evaporated under argon /15/. Aliquots of the lipid residue were analyzed for total phospholipid phosphorus and fractioned to discriminate cholesterol, free fatty acids and the individual spots of the polar lipids as described previously /22,50/.

Lipid extraction from the lens

Immediately after the lens material had been obtained, lipids were extracted from the lens by the Folch method /70/. The extraction was carried out by tissue homogenization in 20 volumes of chloroform/ methanol mixture (2:1 by vol) with BHT antioxidant addition (0.5 mg/100 ml) for 10 min. After filtration the sample obtained was put into a separating funnel for 5-8 h to stratify. Water was added at a 7:1 ratio to promote the stratification. Temperature was maintained at 0°C for all operations. After separation of the phases and removal of the aqueous-methanol layer, the lower chloroform fraction was evaporated. Phospholipid content was assessed by the results of organic phosphorus evaluation /71/. Total lipid amount in the extract was determined gravimetrically, as well as by characteristic absorption at 206-210 nm of the lipid sample after dissolution in 4 ml of methanol/heptane mixture (5:1 by vol).

Determination of lipid peroxidation products

Accumulation of the LPO primary molecular products was estimated spectrophotometrically from characteristic absorbents of diene conjugates in the UV-region at 232 nm characterizing the level of hydroperoxides of polyunsaturated fatty acids, as well as by LPO

secondary molecular products absorbance at 274 nm, corresponding to the concentration of conjugated trienes and cetodienes /72/ on a HITACHI-557 spectrophotometer (Japan). The absorption spectra were recorded after chloroform-methanol (2:1, v/v) extraction as described above and the dissolution of the dry lipid residue in 2.5 ml of methanol-heptane (5:1, v/v) mixture. This protocol removes any water-soluble secondary oxidation products, leaving them in the methanol-aqueous phase. The equalization of the extracted lipid concentrations to the measured phosphorus was done using the characteristic absorption at 206-210 nm of the lipid sample. The content of the source material in the samples was also equalized by the amount of phospholipids. An average molecular weight of phospholipid was assumed as ~730 Da. The content of end molecular fluorescent LPO products was determined from the fluorescence intensity of the lipid extract at 365 nm excitation and 420-440 nm emission wavelengths /73/, measured on a HITACHI-MPF-4 spectrofluorometer. The spectrofluorometer was calibrated at the beginning of every working day against a solution of quinine sulfate (1 μg/ml in 0.1 N H_2SO_4) standard, at 435 nm fluorescence emission and 365 nm excitation wavelengths.

Gas chromatography of halogen-substituted derivatives of the fatty acids

Content of the polyunsaturated fatty acids in the lens is rather moderate, hence direct registration of their decrease in the course of LPO is quite difficult /74/. However, to register an increase of oxy-products in unsaturated fatty acids directly is of rather greater importance than to reveal a decrease in the acid content. In fact, it is the appearance of the fatty acid oxy-derivatives as LPO in the membrane lipid phase, that is well known, that leads to their destruction /41/. In the applied gas chromatography method we used not methyl esters of fatty acids but their halogen-substituted derivatives. By introduction of a fluorine atom into the fatty acid molecule we succeeded in selective labelling of its functional groups. Using this property, it is possible to determine the change of the number of oxy-groups which gain in content in fatty acids in the course of LPO under both *in vitro* and *in vivo* conditions /15/. Electron-capture detection used in gas chromatography of fluorine-substituted compounds was found to be more sensitive than flame-ionization: the minimum

detectable sample flow in substances with high affinity of the electron, such as fluorine-substituted compounds, for electron-capture detection, is of 10^{-11} g/s; that is why this method is the optimum one to measure even lower levels of oxy-derivative fatty acids in tissue lipid extracts /15/.

For selective determination in the lens lipid fraction of the substances containing oxy-groups, fluorine-substituted derivatives of the fatty acids were obtained. For this purpose, after evaporation in the solvent nitrogen current, 100 μl of hexafluorine/isopropanol-benzol mixture (1:4) and 40 μl of penta-fluorinepropine acid anhydride were added to every sample of the lens lipids; the mixture was then sealed and kept at 60°C for 30 min. After this, the ampoule content was evaporated in nitrogen current, the lipid residue was diluted in 150 μl of isooctane and introduced into a Tracor-560 gas chromatograph (USA) equipped with a Tracor-770 autosampler (USA), FSOT-I capillary column (USA) of 50 m length with 0.25 mm inside diameter and also with an electron-capture detector. Hydrogen was used as the carrier gas, with 1:30 flow split. The following temperature conditions were maintained: initial temperature of 140°C (2 min), final temperature of 280°C (15 min), temperature gradient of 3°C/min. Gas chromatographic determination of halogen-substituted fatty acid derivatives was carried out by utilizing the method of inner standard by retention time comparison with the standards. "Sigma" fatty acid standards were used in this study.

Pharmacokinetics of topical *N*-acetylcarnosine application

Formulations and animals

Grey chinchilla rabbits (male) aged 3-4 months weighing 2-3 kg were used. Animal experiments conformed to the guidelines of the ARVO Resolution on the Use of Animals in Research. Thirty min prior to ocular incision the right eyes of the rabbits were instilled with 80 μl of formulation A containing 1% *N*-acetylcarnosine (NAC) and the control right eyes of separate rabbits were similarly instilled with vehicle (placebo) solution. Formulation A (Can-C™) contained the following ingredients: deionized water 970 g, 1.0% glycerine 13 g, 1.0% NAC 10 g, 0.3% carboxymethylcellulose 3 g, 0.3% benzyl

alcohol 3 g, potassium borate 7.91 g*, and potassium bicarbonate 3.47 g*.

Formulation A was present in the final ophthalmic tubes (per volume of 2.5 ml) and in the moiety of the plastic bottles. Placebo (Formulation B) solution contained the same ingredients without NAC. The solution of NAC in the phosphate buffer was also administered in the right eyes of the rabbits. The following formulations were used: Formulation C: NAC dissolved in sodium phosphate solution, pH 6.3. This was the research 1% NAC formula with the preservative thimerosol added at 0.004 g per 100 ml. Formulation D: (1% NAC was added) benzyl alcohol dissolved in the sodium phosphate buffer, pH 6.3, and at the same dissolution rate. Formulation E: (1% NAC was added) phenyl ethyl alcohol dissolved in the sodium phosphate buffer, pH 6.3, and at the same dissolution rate.

Surgical procedure

Topical anaesthesia of rabbit eyes was performed after 25 min of instillation of the formula ophthalmic solutions with instillations of 4% lidocaine hydrochloride solution eye drops (three times with 1 drop at 1.5-2.0 min intervals). The eye drops of 4% lidocaine hydrochloride contain benzaltonium chloride preservative. When ocular anaesthesia was achieved, the lids were extended and fixed with the lid-holder and the ocular bulb was fixed by tweezers in the area of the inferior rectus muscle. A stab incision was performed transcorneally 1.0-2.0 mm from the limbus in the temporal superior quadrant. Aqueous humor (0.1-0.2 ml) was aspirated from the anterior chamber of the rabbit's eye with a 25-gauge needle connected to an insulin syringe and immediately introduced into an Eppendorf tube with the addition of ethanol (0.2 ml), keeping the sample on ice before extraction.

Extraction of imidazoles from aqueous humor

Extractions of imidazole-containing compounds from the aqueous humor aliquots were performed according to Babizhayev *et al.* /60/. Portions of aqueous humor were added to ethanol as above and thoroughly mixed (20°C, 15 min). Extracts were centrifuged (2000 *g*,

* or whatever was necessary to bring the solution to pH ~6.3-6.5.

15 min) and the supernatants removed .Samples were frozen in the gradient of temperatures to -70°C and lyophilized using the apparatus JOAN (France). The lyophilized residue was dissolved in 1 ml of 0.1 M Na_2HPO_4 (pH 2.1 adjusted with 85% phosphoric acid) and filtered through a membrane with pore dimension 0.22 μm immediately prior to analysis.

Analytical HPLC

Reverse phase analytical HPLC was performed using a Breeze chromatography system (USA), Waters 2487 dual λ absorbance detector, Symmetry 300 column (250 x 4.6 mm) C18 5 μm (Waters), loop 20 μl. The column was eluted isocratically at 30°C with the cited phosphate buffer 0.1 M Na_2HPO_4 (pH 2.1) over 25 min at a flow rate of 1.0 ml/min. Eluates were monitored for absorbance at 210 nm. Standards of L-carnosine and *N*-acetylcarnosine were prepared by weighing dry material using a Mettler Toledo analytical balance (accuracy 0.00004) and were further dissolved in phosphate buffer 0.1 M Na_2HPO_4 (pH 2.1). The quantitative determination of L-carnosine and *N*-acetylcarnosine in the samples was undertaken using the technique of external standard according to the area of the peak and linear extrapolation. Standards of eye drops were prepared by dissolution of initial solutions of eye drops 100-fold using phosphate buffer 0.1 M Na_2HPO4 (pH 2.1).

Statistical significance was evaluated using Student's unpaired t-test, and $p = 0.05$ was taken as the upper limit of significance.

Peroxidation reaction system

The techniques for phospholipid extraction, purification and preparation of liposomes (reverse-phase evaporation technique) have been described previously /44,50/. Peroxidation of phosphatidylcholine (PC, derived from egg yolks) was initiated by adding 2.5 μM $FeSO_4$ and 200 μM ascorbic acid to the suspension of liposomes (1 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.4). The incubations were performed at 37°C. The tested compounds, NAC and L-carnosine, were added at 10-20 mM concentration to the system of iron-ascorbate-induced liposome PC peroxidation. The kinetics of accumulation of lipid peroxidation (LPO) products in the oxidized liposomes were measured by reaction with thiobarbituric acid (TBA). The

peroxidation reaction was arrested by adding EDTA to a final concentration of 50 μM or by the addition of 2.0 ml of ice-cold 0.25 M HCl containing 15% (w/v) trichloroacetic acid (TCA). TBA (0.125% w/v) was then added to the mixture and followed by boiling for 15 min. The TBA assay was described previously /50/. The differential absorbance of the condensation product, malonyl dialdehyde (MDA), at 535 and 600 nm was measured spectrophotometrically (ε 535 = 1.56 x 10^5 $M^{-1} \cdot cm^{-1}$). The TBA reaction itself was not affected by the components of the radical generators or scavengers used in the study.

To determine conjugated dienes the lipid residue of the samples was partitioned through chloroform during the extraction procedure /50/. Correlation of the extracted lipid concentrations to the measured phosphorus was done by means of characteristic absorption at 206-210 nm of the lipid sample (redissolved in 2-3 ml of methanol/heptane mixture, 5:1, v/v). Accumulation of net diene conjugates corresponding to the level of lipid hydroperoxides was assessed from characteristic absorbance of diene conjugates at ~230 nm (ηCD = 2.8 x 10^4 $M^{-1} \cdot cm^{-1}$) in a Shimadzu UV-260 spectrophotometer (Japan) /50/. Absorbance of the secondary LPO products at ~274 nm, corresponding to the concentration of conjugated trienes and ketodienes, was also measured spectrophotometrically from the lipid spectra /50/.

Statistical significance was evaluated by Student's unpaired t-test, and $p = 0.01$ was taken as the upper limit of significance.

Uptake of L-carnosine by the lens

In a separate series of experiments, the kinetics of L-carnosine penetration into isolated rabbit lenses was evaluated (five lenses studied). To assess the ability of the lens to accumulate L-carnosine, it was placed in 5 mM (or higher concentration) L-carnosine solution in Hanks' salt medium (without bicarbonate, pH 7.4), containing 7 mM glucose /44/ and incubated at room temperature (20°C). After 1 h, the extraction procedure was utilized /44/ to isolate L-carnosine from the non-protein fraction of the lens. The non-protein fraction removed was stained with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-chloride) reagent and then applied for TLC in aqueous ethanol ($EtOH/H_2O$, 77:23). The concentration of L-carnosine in the sample was estimated spectrophotometrically from its characteristic absorbance at 420 nm/600 nm in a Hitachi-557 double-beam spectrophotometer (Japan).

Standard L-carnosine samples were used to evaluate the quantitative recording of the absorbance. TLC was applied earlier /54/ to evaluate the L-carnosine level in eye lenses.

Incubation of lenses with liposomes

To control effects of metal ions or potential OH· scavengers (such as glucose present at scavenging concentrations in standard culture media), the lens culture medium was composed of 10 mM Tris-HCl, 140 mM NaCl (pH 7.4), phosphate- and glucose-free (Medium A), or glucose-containing medium nutritious to the lens comprising 50 mM Tris-HCl, 5.5 mM glucose, 4.0 mM KCl, 102.5 mM NaCl, 1.0 mM K_2HPO_4 (pH 7.2-7.4) (Medium B) /50/. Both media were adjusted to 290-300 mOsmol with NaCl and equilibrated with 95% air and 5% CO_2. When significant concentrations of oxygen scavengers or other agents were added, the change in osmolarity was compensated by decreasing the amount of NaCl used to adjust the media back to the required osmolarity following modification. The rabbit lenses were incubated in 3.0 ml of medium per lens at room temperature. The tests of lens integrity during incubations, to indicate that the lens was functioning normally, were performed as previously reported /75/. When necessary, the lens incubation media contained the liposome suspension (0.5 mg/ml). Fixed aliquots of media (50-500 μl) were taken out at different times from the organ cultures of lenses for measurements of LPO products. Generally, the total incubation time was 3 h.

RESULTS

Aqueous humor

Lipid and phospholipid contents in aqueous humor samples obtained from human eyes were reported recently /50/. Typical UV absorption spectra of lipids have their maximum in the 206-nm regions related to absorption of isolated double bonds of hydrocarbon phospholipid chains (Fig. 2). Lipid extract from normal aqueous humor exhibits a modest shoulder of absorption at about 230 nm and a certain absorption peak around 280 nm in the UV region. However, lipid extract of patients with cataract usually exhibits the stronger

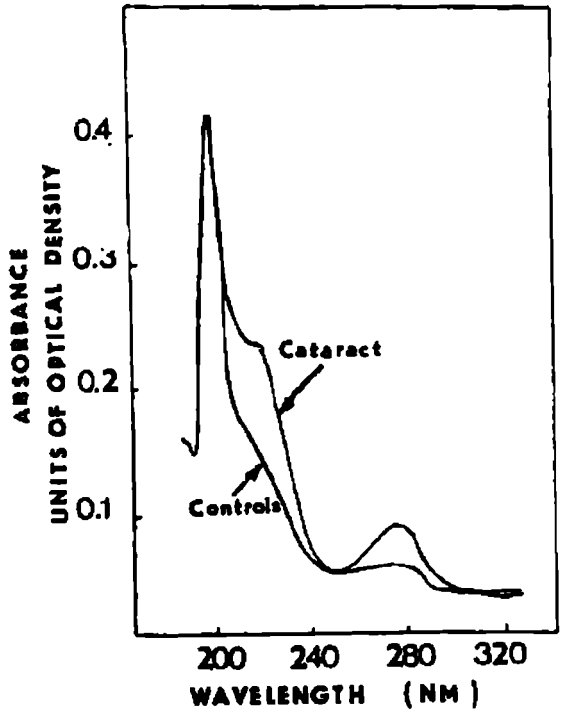


Fig. 2: UV absorption spectra of lipid extracts (methanol-heptane 5:1, v/v) from the aqueous humor samples, obtained from human eyes: normal (control, lower curve) and mature cataract (upper curve). See Methods for details of processing procedure.

absorption shoulder at 230 nm, characteristic of conjugated double bonds in the fatty acids (primary LPO products, lipid hydroperoxides) and an absorption peak around 280 nm (characteristic of ketones, aldehydes, secondary molecular LPO products) (Fig. 2, upper curve). When the level of secondary products not removed into the water-soluble phase was not significantly increased in UV absorption spectra of lipid extracts of aqueous humor from the cataractous patients (Table 1), the distinct increase of the contents of the end fluorescent LPO products expressed in terms of arbitrary fluorescence units/μg phospholipids (PL) was about 2.4-fold and 4.2-fold higher correspondingly for immature or mature cataracts as compared to normal

TABLE 1

Lipid peroxidation products in aqueous humor from the eyes of patients with cataract

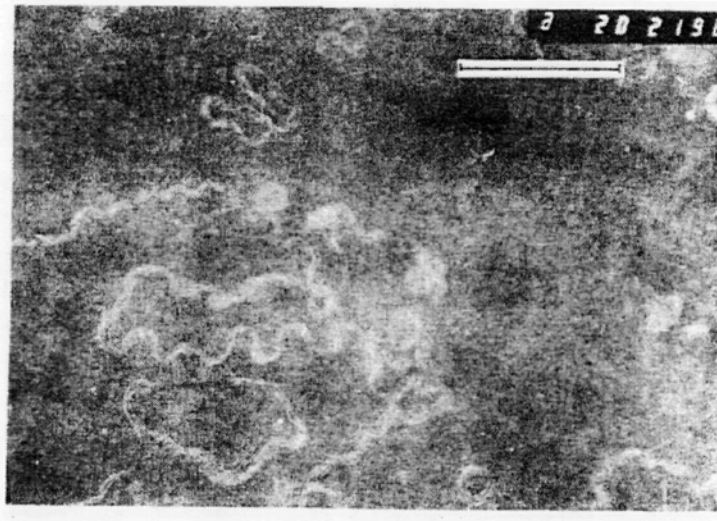
Lipid peroxidation products	State of the lenses/degree of lens clouding		
	Control (0.0-10%) (n = 10)	**Immature cataract** (10-64%) (n = 12)	**Mature cataract** (64-100%) (n = 16)
I. Diene conjugates			
1. OD_{232}/mg phospholipids	5.86 ± 0.44	7.93 ± 0.65 [b]	9.48 ± 0.34 [c]
2. OD_{232}/OD_{206}	0.307 ± 0.024	0.425 ± 0.035 [d]	0.518 ± 0.020 [c]
3. mole/100 mole phospholipids	2.37 ± 1.14	5.40 ± 1.70	9.44 ± 0.89 [c]
II. Triene conjugates, ketone and aldehyde products			
1. OD_{274}/mg phospholipids	2.33 ± 0.13	2.39 ± 0.23	2.86 ± 0.40
2. OD_{274}/OD_{206}	0.122 ± 0.007	0.133 ± 0.012	0.163 ± 0.022
III. Fluorescence products			
Fl/μg phospholipids	17.2 ± 6.4	42.0 ± 7.3 [b]	71.7 ± 20.2 [b]

Data are means ± SD; n = number of examined samples.
Significant differences from control: [b] $p < 0.05$, [c] $p < 0.001$, [d] $p < 0.02$.

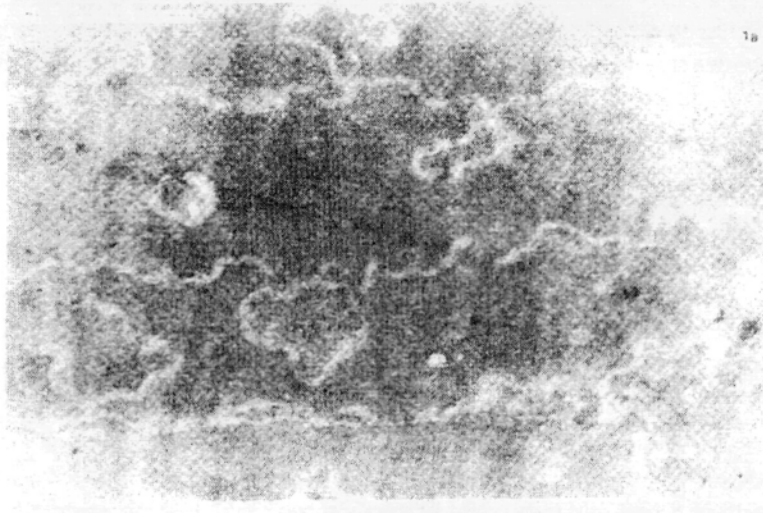
lenses (Table 1). In the samples of aqueous humor aspirated from donor eyes with normal lenses, one in about 42 PL molecules contains a conjugated diene, while in case of immature cataract (IDLC 10-64%), the average amount of conjugated dienes reaches one in 19 aqueous PL molecules, whereas in mature cataract (IDLC 64-100%) this estimation averages one conjugated diene per 11 PL molecules. Net concentrations of primary (diene conjugates, phospholipid hydroperoxides) and end (fluorescent) LPO products were found to be increased in aqueous humor of the anterior chamber in cases of cataract irrespective of its genesis comparated to donor (control) eyes and in line with the maturity stage measured by degree of clouding (Table 1).

Changes in ultrastructural organization of the lens fiber membrane integrity during the development of cataract

The electron microscopic observations showed that, when the lens still preserves its transparency and protein aggregates cannot be detected in its tissue, the earliest changes in regular longitudinal lens fiber plasma membrane structure, did occur (Fig. 3a). They form typical 'knobs' and 'sockets' (Fig. 3b). Consequently, lens opacification is preceded by deterioration of the lenticular fiber plasma membrane ultrastructure. Following opacification progression the lens fibers become more irregular, their electron density increases, and the membranes form numerous diverticula, twistings, interdigitations, and fragments (Fig. 3c). Then further membrane fragmentation and an increase of the plasma membrane fragments' curvature are observed. Figure 3d shows typical 'wave-like' membrane structures forming multiple whorls around electron-dense centers. Such undulating structures differing by a number of different forms expand eccentrically from the lens fiber. Such formation has a 'string of pearls'-like shape. At the mature stage of cataract (Fig. 3e), which is biochemically characterized by large high molecular aggregate formation in the human lens, ultrastructural characteristics of the membrane lesion are distinct with lenticular fiber plasma membrane fragments becoming twisted into a central mass of amorphous electron-grey debris and globules of different size (100-900 nm) and electron-dense granular contents. At the same time, fiber and membrane structures could no longer be identified and fibers appeared to have been converted to extensive masses of globular structures. First, smaller

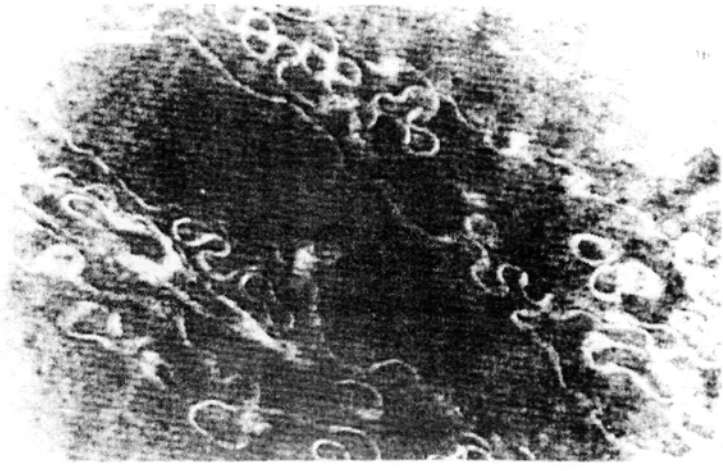


a

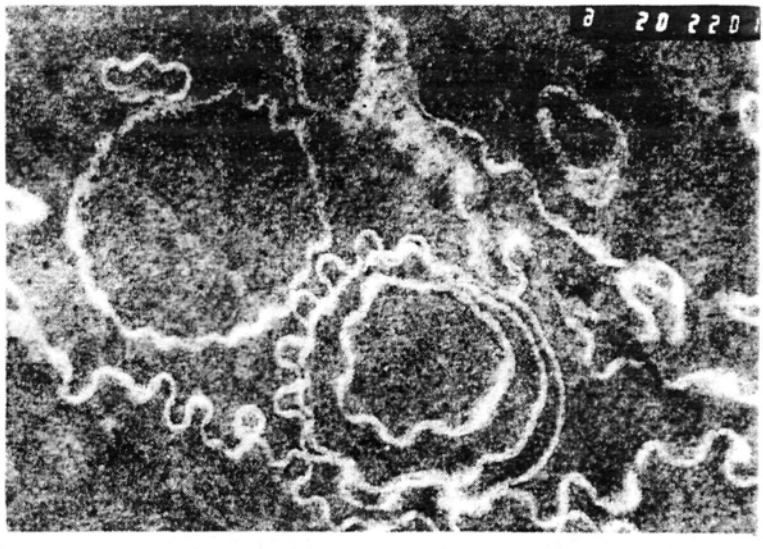


b

Fig. 3: **a.** Electron micrograph of superficial posterior cortex of the cataractous human lens showing apparently normal lens fibers exhibiting a moderate degree of interdigitation lying outside the cataract zone. **b.** Electron microphotograph of the midzone area of the normal human lens. Changes in regular longitudinal lens fiber plasma membrane structure form typical 'knobs' and 'sockets'. Bars = 1 μm.

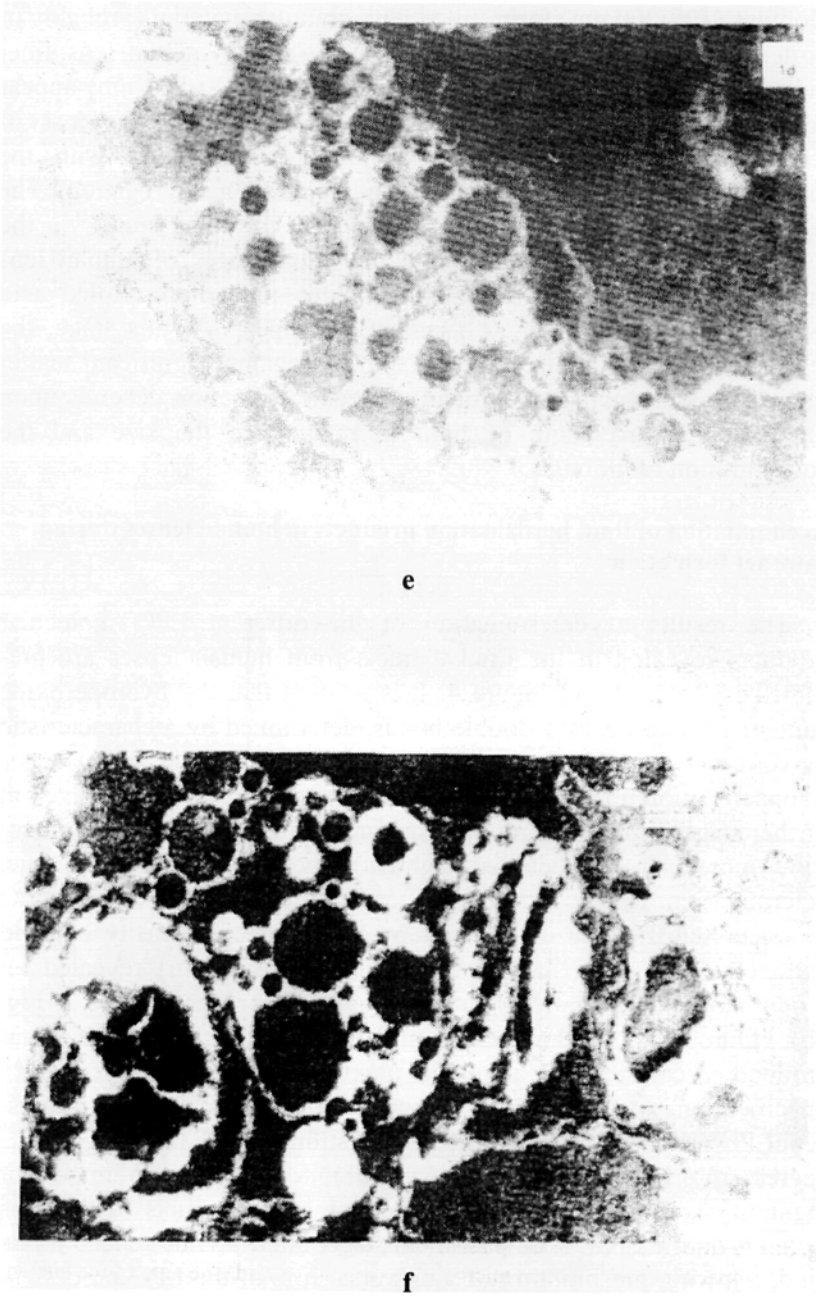


c



d

Fig. 3: **c.** Modification of the plasma membranes of the lens fiber cells by lens opacification. Electron microphotograph of the midzone area of the human lens. Immature cataract. Bar = 1 μm. **d.** Typical 'wave-like' membrane structures forming multiple whorls around electron-dense centers. Scale as in **c**.



e

f

Fig. 3: Electron microphotograph of the midzone area of the human lens. Bar = 1 μm. **e.** Nuclear cataract. **f.** Mature cataract.

globules (100-200 nm) form, filled with granular material (probably of protein nature) of about the same density as the human lens fiber cytoplasm, and then intermediate size globules (220-500 nm) appear containing aggregates of high density and disparity. The loss of lenticular fiber-free membranes is probably connected with the mentioned membrane involvement in the protein aggregation. The larger globules (≥600 nm) seem to develop from the small via the intermediate globules (Fig. 3f). At the latter stages of human lens opacification, the globules become more and more coiled and aggregated, and intermediate filaments disappear. At this stage, the lens matter is filled mainly with protein aggregates. Significant scatter of light accompanying the protein aggregate formation depends upon the incident wavelength of light in relation to the size and the concentration of globules.

Accumulation of lipid peroxidation products in human lenses during cataract formation

The results of determination of the different LPO molecular products revealed in the lipid extracts from human lenses are presented in Table 2 and Figure 4. It is evident that the hydroperoxide content with conjugated double bonds, determined by a characteristic maximum in the UV spectrum at 230 nm, increases at the initial stages of opacification up to the almost mature stage of cataract. However, at further stages of mature and hypermature cataract, the level of primary LPO molecular products is slightly reduced. At the same time, determination of the content of end molecular fluorescent LPO products-Schiff bases determined by fluorescence intensity of lipid extract at 430 nm (fluorescence excitation, 365 nm) revealed an monotonous increase along with cataract development (Table 2, Fig. 4b). Figure 4a shows that the accumulation of primary LPO molecular products in cataract lens attains its maximum at the stage of 55-64% opacification. At the same time, the concentration of the end fluorescent LPO products in the lens correlated strongly with the integral lens degree of opacity ($r = 0.956$, $p < 0.01$). Meanwhile, an important regularity was revealed: accumulation of LPO products in the lens depends on the cataract development stage, but does not depend on its kind, allowing presumption of a universal role of the LPO process in lens opacification.

TABLE 2

Content of lipid peroxidation products in human lenses

Cataract stage	α (%)	n	C (AU)	OD_{232}/OD_{206}	C′ (AU)	OD_{274}/OD_{206}	Fl
Transparent lens	0	18	1.9 ± 0.2	0.30 ± 0.02	0.45 ± 0.05	0.07 ± 0.01	18 ± 5
Incipient	<10	6	2.4 ± 0.2 p <0.1	0.37 ± 0.03 p <0.1	0.58 ± 0.10 p <0.1	0.09 ± 0.01 p <0.1	19 ± 7 p >0.1
Immature	10-55	36	2.5 ± 0.1 p <0.01	0.39 ± 0.015 p <0.01	0.67 ± 0.05 p <0.01	0.13 ± 0.01 p <0.01	45 ± 8 p <0.02
Almost mature	55-64	26	2.9 ± 0.2 p <0.01	0.45 ± 0.03 p <0.01	0.80 ± 0.06 p <0.01	0.16 ± 0.01 p <0.01	53.4 ± 12.0 p <0.02
Mature	64-100	53	2.4 ± 0.1 p <0.05	0.36 ± 0.025 p <0.1	0.66 ± 0.06 p <0.02	0.11 ± 0.01 p <0.1	74.1 ± 13.2 p <0.01
Hypermature	~100	9	2.2 ± 0.2 p >0.1	0.35 ± 0.04 p >0.1	0.45 ± 0.07 p >0.1	0.08 ± 0.01 p >0.1	97.8 ± 10.0 p <0.01

Data are means ± SD.

α = lens opacity; C = diene conjugates concentration; OD_{232}, OD_{274}, OD_{206} = optical density at 232, 274, 206 nm, respectively; C′ = triene conjugates (cetodienes) concentration; Fl = fluorescence intensity of lipid extract in relative units; n = number of examined lenses; AU = arbitrary units.

p <0.05: significant compared to transparent lenses.

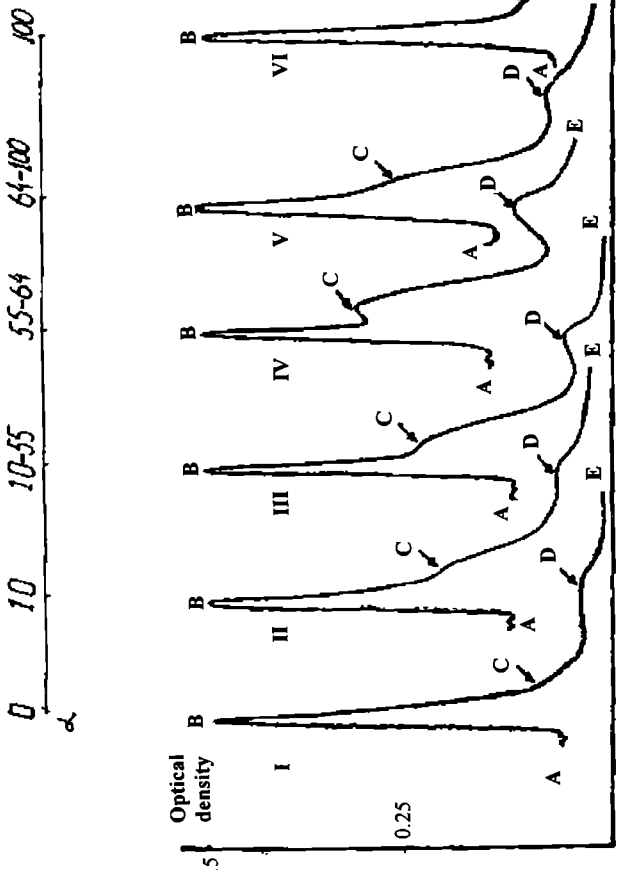


Fig. 4a: The dynamics of accumulation of primary molecular lipid peroxidation products during opacification of the human lens. A = % lens opacity; I-VI = stages of human cataract; A: 190 nm, B: 206 nm, C: 230 nm (diene conjugates maximum), D: 274 nm (triene conjugates maximum), E: 330 nm.

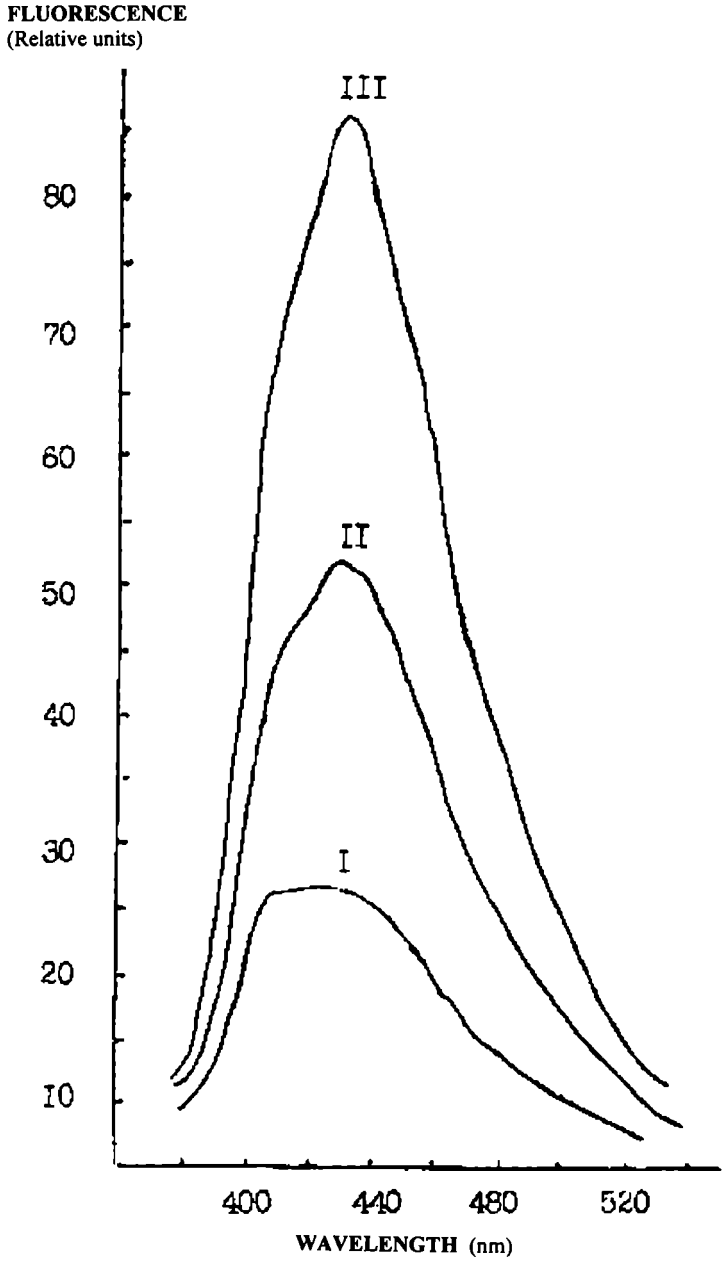


Fig. 4b: Characteristic fluorescence spectra of lipid extracts from human lenses by excitation wavelength of 365 nm. I: normal; II: almost mature cataract; III: mature cataract.

The study of halogen-substituted derivatives of fatty acids in the human lens

Typical chromatograms of lipid fraction from transparent and cataractous (mature cataract) human lenses are shown in Figure 5. Although in mature cataract, in the gas chromatographic profile of the lens lipid sample, no principal new peaks are found to appear, the intensity of peaks differs markedly from the norm as a quantitative ratio. The 'A' peak corresponds to the fatty acid 18:1. As this method allows the determination of both fatty acids and their oxidized metabolites, in the absence of a complete set of standards, no identification of all the chromatographic peaks was performed in the present study. However, a reliable increase in the intensity of the peaks whose retention time does not correspond to fatty acids non-oxidized standards (Table 3) most probably reflects the fact of the increase in content of oxy-derivative polyunsaturated fatty acids in the cataract.

***N*-Acetylcarnosine as a time release (carrier) version of L-carnosine in ophthalmic applications with carboxymethylcellulose**

An important chemical difference between carnosine and *N*-acetylcarnosine is that carnosine is relatively insoluble in lipids (fats and fatty compounds), whereas *N*-acetylcarnosine is relatively soluble in lipids (as well as in water). This means that *N*-acetylcarnosine may pass through the lipid membranes of the corneal tissue and cellular structures more easily than carnosine, and may thereby more readily gain access to the intraocular aqueous humor. *N*-Acetylcarnosine can gradually release carnosine which then exerts its beneficial effects /60/. In the present section of the study, we considered whether NAC acts in the ophthalmic formulation with lubricants (including carboxymethylcellulose) and preservatives when topically administered to the eye as a time release carrier (prodrug) of L-carnosine. The HPLC pattern of an extract of the aqueous humor obtained 30 min after instillation to the rabbit eye of ophthalmic formulation containing 1% NAC, lubricants carboxymethylcellulose and glycerine, and preservative benzyl alcohol in borate buffer confirms that the peak characteristic of L-carnosine has a concentration and a retention time (3.1 min) clearly distinct from *N*-acetylcarnosine (6.0 min) and from the dead time of the column (Fig. 6). This identified peak of L-carnosine was quantified and the data processor integrated that virtually all *N*-

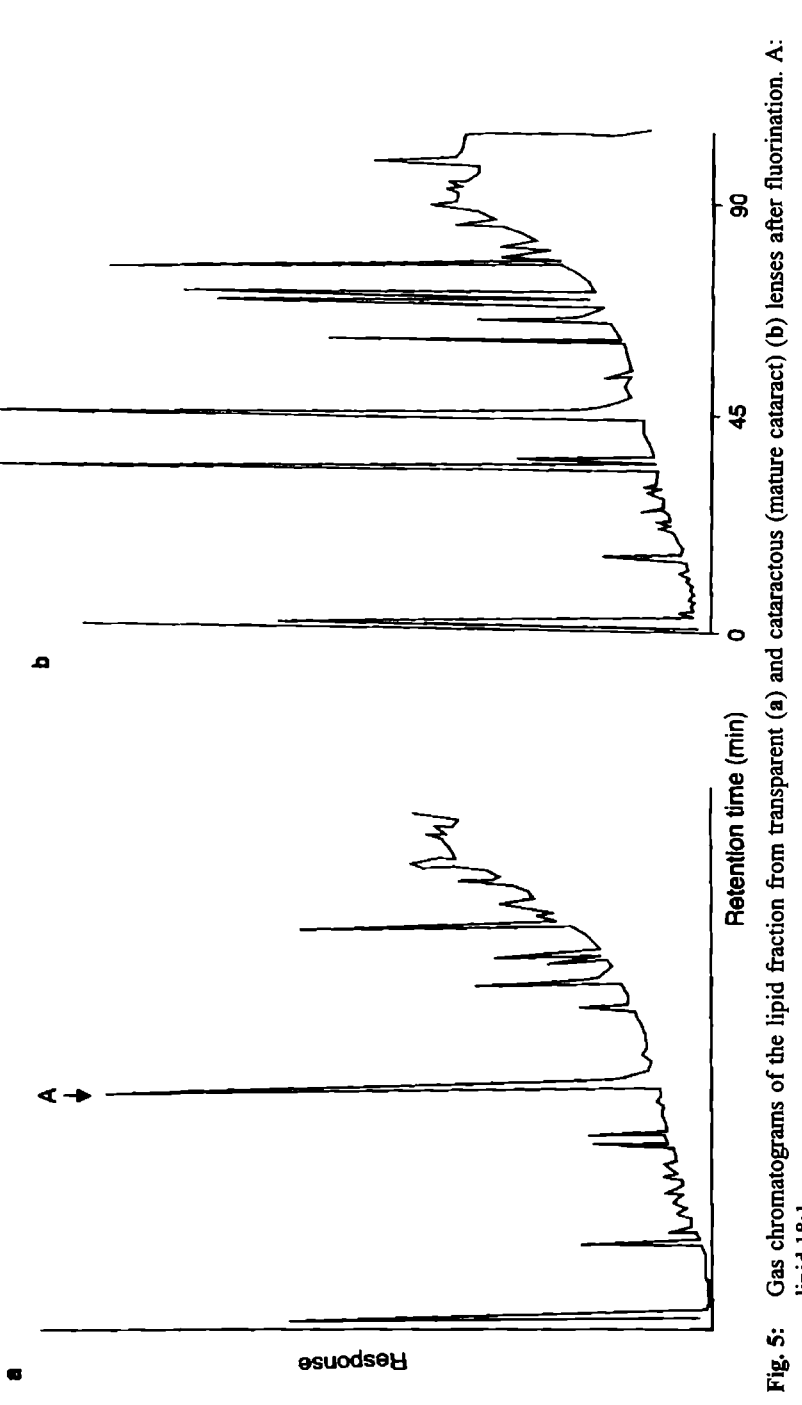
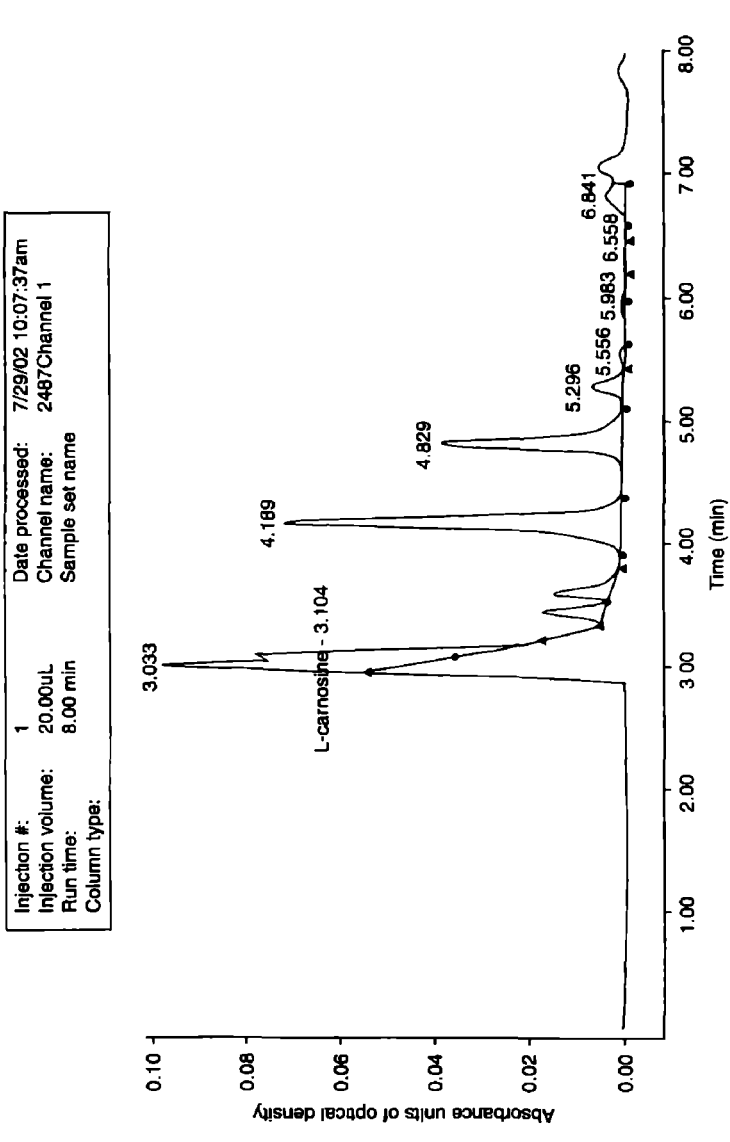


Fig. 5: Gas chromatograms of the lipid fraction from transparent (a) and cataractous (mature cataract) (b) lenses after fluorination. A: lipid 18:1.



	Peak name	RT (min)	Area (µV*sec)	Area (%)	Height (µV)	Height (%)	Amount	Units
1		3.033	251 219	17.42	52 493	21.24		
2	L-Carnosine	3.104	186 774	12.95	45 509	18.42	7.079	ug/mL
3		3.453	64 253	4.46	12 971	5.25		
4		3.610	67 747	4.70	11 790	4.77		
5		3.880	1 270	0.09	359	0.15		
6		4.189	493 858	34.24	71 709	29.02		
7		4.829	269 502	18.69	38 783	15.70		
8		5.296	43 914	3.04	6 341	2.57		
9		5.556	5 970	0.41	1 041	0.42		

Fig. 6: HPLC of extract of aqueous humor aspirated 30 min after the instillation of ophthalmic formulation with 1% *N*-acetylcarnosine and lubricants into rabbit eye.

TABLE 3

Analysis of fluorinated derivatives of fatty acids in transparent and cataractous human lenses

Retention time (min)	Concentration (%)		p
	Transparent lens (n = 5)	Mature cataract (n = 7)	
17.32	2.08 ± 0.10	0.65 ± 0.10	<0.01
35.26	2.17 ± 0.10	10.89 ± 1.10*	<0.01
35.76	2.34 ± 0.10	1.55 ± 1.00	>0.1
45.61	42.6 ± 1.1	40.7 ± 1.1	>0.1
59.83	1.27 ± 0.10	2.83 ± 0.10*	<0.01
63.67	19.56 ± 1.00	0.70 ± 0.10	<0.01
67.68	1.6 ± 0.1	0.6 ± 0.5	>0.1
68.7	2.61 ± 0.10	4.0 ± 0.1*	<0.01
73.93	2.0 ± 0.1	7.1 ± 0.1*	<0.01
74.35	9.1 ± 0.1	0.6 ± 0.1	<0.01
78.07	1.8 ± 0.1	0.6 ± 0.2	<0.01
82.17	1.8 ± 0.2	0.6 ± 0.2	<0.01
84.95	1.4 ± 0.1	0.6 ± 0.2	<0.01
85.37	5.7 ± 0.1	0.6 ± 0.3	<0.01
86.1	2.1 ± 0.1	0.6 ± 0.2	<0.01
94.58	1.8 ± 0.2	2.2 ± 0.2*	>0.1

* Chromatographic peaks characterizing fatty acid oxyproducts accumulation. Oxyproducts accumulation ratio in comparison with transparent lens is 17.2%.

acetylcarnosine after the overall extraction efficiency is converted into L-carnosine with a retention time of 3.1 min (Fig. 6). The data on the L-carnosine-related product were blanked with the control placebo data applied to the matched eyes of the control rabbits. The mean ratio of L-carnosine (C)/NAC relevant to L-carnosine release in the aqueous humor 30 min after instillation of Formulation A with 1% *N*-acetylcarnosine into the rabbit eye corresponded to C/NAC = 6.64 ± 0.06 (n = 8, where n = number of the examined treated rabbit eyes; only right eyes were treated). In the control placebo formulation-treated eyes the same indices could not be quantified at a statistically significant rate. Concentrations of imidazole products in the aqueous humor (0.19 ± 0.10 μg/ml) corresponded to those of intact rabbit eyes and refer to baseline values of L-carnosine-related products variously detected in extracts from normal animals. When we used control formulations containing only 1% *N*-acetylcarnosine dissolved in a buffered solution, or 1% *N*-acetylcarnosine and added preservative (thimerosol, benzyl alcohol or phenyl ethyl alcohol) dissolved in a buffered solution (Formulations C-E, see Methods), the C/NAC ratio corresponded to 1.99 ± 0.05 (n = 7), 1.94 ± 0.12 (n = 5), 1.98 ± 0.05 (n = 5), 1.95 ± 0.05 (n = 5), respectively. Our data demonstrate that topical administration of pure L-carnosine (1% solution) to the rabbit eye (instillation, subconjunctival injection) does not lead to accumulation of this natural compound in the aqueous humor over 30 min in concentration exceeding that in the placebo-treated matched eyes, and its effective concentration is exhausted more rapidly /60,61/. The *N*-acetylcarnosine prodrug conversion to the active drug (L-carnosine) in the aqueous humor, enhanced by the mucoadhesive compound and corneal absorption promoter present in the ophthalmic formulation, takes place in the corneal and conjunctival tissues when the *N*-acetylcarnosine ingredient passes from the cornea to the aqueous humor. Detailed consideration of the drug's penetration into the aqueous humor and the lens promoted by various cellulose-containing compositions including *N*-acetylcarnosine has been published recently /76/. From another aspect, the data demonstrate a method for the prevention or treatment of an eye disease, comprising topical application to a mammal in need of treatment of an aqueous ophthalmic composition comprising *N*-acetylcarnosine or a pharmacologically acceptable salt of *N*-acetylcarnosine, in combination with a lubricant

(cellulose compound) in an amount effective to increase intraocular absorption of L-carnosine into the aqueous humor.

Antioxidant activity of *N*-acetylcarnosine versus L-carnosine in the liposome peroxidation system

The comparative antioxidant activity of NAC and L-carnosine was assessed in the liposome peroxidation system catalyzed by Fe^{2+}-ascorbate (Fig. 7). The accumulation kinetics of molecular LPO products, such as MDA and liposomal conjugated dienes and trienes, is shown in Fig. 7. The results demonstrate that the LPO reactions in the model system of lipid membranes are markedly inhibited by L-carnosine. The effective concentrations of L-carnosine are 10 and 20 mM. Data on the biological effectiveness of L-carnosine as an antioxidant preventing PC liposomal or linoleic acid peroxidation in physiological concentration ranges of 5-25 mM have already been published /44,50,52/. Figure 7A shows that the level of TBA reactive substances (TBARS) reached at 5-min incubation decreases in the presence of L-carnosine (10 or 20 mM) at 10 min and at later time points (20 mM), which must be due to a loss of existing TBARS or peroxide precursors of MDA and not due to a decreased formation of peroxide compounds. The ability of the histidine-containing compound NAC to inhibit the Fe^{2+}ascorbate-induced oxidation of PC liposomes was compared with that of equimolar concentrations of L-carnosine. The antioxidant activity of 10 and 20 mM NAC corresponded to 38% and 55% inhibition of LPO for the two concentrations after 60 min incubation. NAC exhibited less antioxidant protection than L-carnosine, corresponding to 60% and 87% of the equimolar (10 or 20 mM) L-carnosine inhibition percentage. However, since NAC can act as a time release prodrug metabolized into L-carnosine during its passage through the cornea to the aqueous humor (but not oral use), the antioxidant activity of NAC *in vivo* application is significantly increased. Once released from NAC, L-carnosine in the aqueous humor might act against peroxidation of the lens during its target pharmaceutical use.

The biological effectiveness of L-carnosine as a specific scavenger for activated oxygen species was assessed in the crystalline lens-induced LPO system. Transparent rabbit lenses were incubated in the various media containing 0.5 mg/ml liposome suspension as the oxidation substrate, and the kinetics of the LPO reaction were

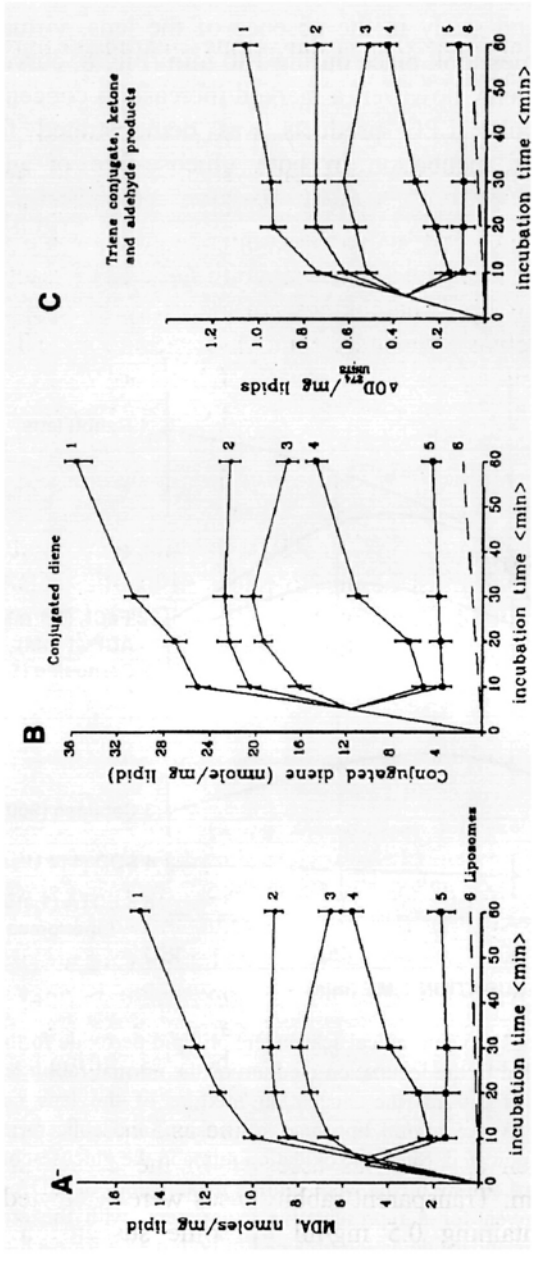


Fig. 7: Accumulation of lipid peroxidation products (TBARS, measured as MDA) (**A**), diene conjugates (**B**), triene conjugates and ketone and aldehyde products (274 nm absorbing material) (**C**) in liposomes (1 mg/ml) incubated for 60 min alone (6, dotted line) and with addition of the peroxidation-inducing system of Fe^{2+}ascorbate (line 1). Antioxidants *N*-acetylcarnosine (NAC) (10 or 20 mM) (lines 2, 3) or L-carnosine (10 or 20 mM) (lines 4, 5) were added at the fifth minute of the incubation period to the system containing the peroxidation inducers. Samples were taken at zero time and at time intervals indicated in the figures and were used immediately for measurement of TBARS (see Methods). A similar amount of sample was partitioned through chloroform and used for detection of conjugated dienes and trienes dissolved in 2-3 ml of methanol-heptane mixture (5:1 v/v).

estimated by measuring MDA and liposomal conjugated dienes and trienes making appropriate corrections for liposome autooxidation /50/. In a background study in the absence of the lens, virtually no oxidation of liposomes took place during 180 min (Fig. 8, curve 6). In the presence of the lens, however, a marked increase in concentration of different molecular LPO products was demonstrated for the appropriate time of incubation in both glucose-free or glucose-

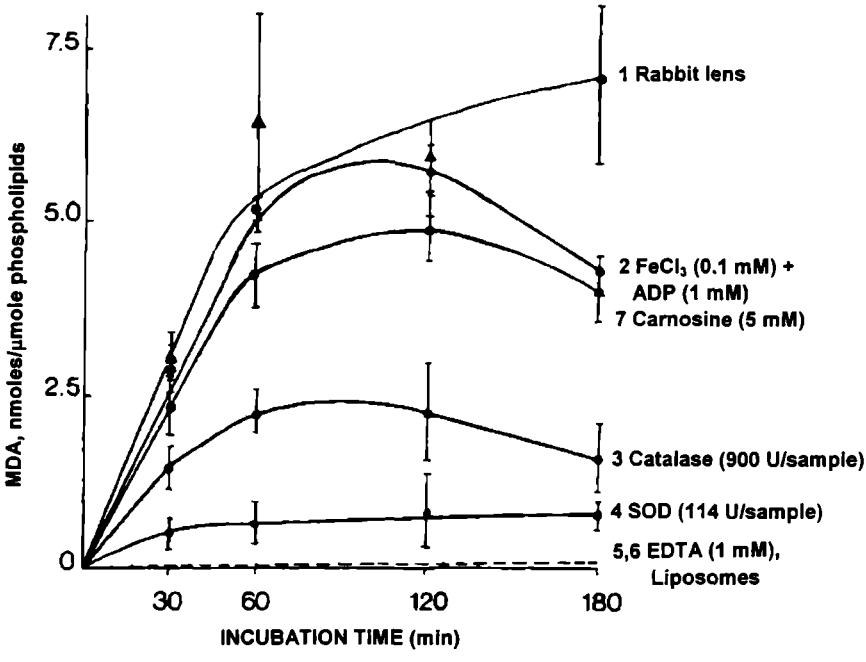


Fig. 8: Effect of various oxygen radical scavengers on lipid peroxide formation in liposomes added to the incubation medium of the normal rabbit lens. In a total volume of 3.0 ml the incubation mixture of the lens contained medium A or B, 0.5 mg/ml liposome suspension, and concentrations of scavenger as indicated. Since the oxidation ratios in the glucose-containing or free standard culture media were similar, mean values of MDA concentrations are given for a representative experiment, with the error bars indicating the standard deviation obtained in both media for each group of three to five lenses.

containing culture media (Fig. 8, curve 1) /50/. The level of MDA accumulation after incubation of rabbit lenses for 3 h was 3.5- and 5.3-fold higher than of normal human or mouse lenses. The larger normal lenses (rabbit or human) have more epithelial cells rich in reduced glutathione (GSH), which are metabolically active, and these lenses can generate active oxygen species and lipid peroxides more rapidly than cataractous lenses, with their exhausted pool of reductants, or tiny mouse lenses /50/. In some cases a small decrease in liposomal MDA concentration after incubation for 2 h was observed. This may be connected with MDA utilization by the lens itself (interaction of MDA with amino group, or its lowering by lenticular aldehyde dehydrogenase /50/). A considerable reduction in the accumulation rate of liposomal LPO products was found after the addition of catalase (900 U/sample) (Fig. 8, curve 3). This suggests a role of H_2O_2 in promotion of LPO by the lens. Addition of superoxide dismutase (SOD, 114 U/sample) to the incubation medium of the lens led to a marked reduction of liposomal MDA level (Fig. 8, curve 4), suggesting that the lens can generate $O_2^{-\cdot}$ in the surrounding medium. Addition of the ADP-Fe complex to the incubation medium of the lens decreased accumulation of TBA-reactive material in liposomes by 33-50%, indicating decomposition of the accumulated TBARS. Almost total inhibition of TBA reactivity in the liposomes occurred after the addition of the chelating agent EDTA, 1 mM, which eliminates free and accessible metal ions from the peroxidizing system (Fig. 8, curve 5). L-Carnosine has been shown to act as a good scavenger of the lipid peroxyl (LOO·) and hydroxyl (OH·) radicals /52,77/. The presence of 5 mM L-carnosine in the rabbit lens/liposome-containing medium decreased TBA reactivity by approximately 25% at 2 h incubation (Fig. 8, curve 7).

Study of L-carnosine uptake by the lens

To study the kinetics of L-carnosine penetration into the lens, the isolated rabbit lens was incubated in a medium containing L-carnosine (5-15 mM). Incubation of lens preparations with histidine dipeptide led to its accumulation in the lens tissue. It can be concluded that L-carnosine penetrates the barrier of the lens capsule when present in the aqueous humor at effective concentrations. The presence of L-carnosine in transparent crystalline lenses was detected and its concentration in this case was about 25 μM in normal human lenses

and 0.89 ± 0.1 mM (n = 4) in rabbit lenses /54,55/. At different stages of cataract development, the level of L-carnosine fell, reaching about 5 μM in mature human cataracts /54/. Thus, L-carnosine that finds its way into the aqueous humor can accumulate in the lens tissue for a reasonable period of time.

Synergism of histidyl dipeptides and amino acids as antioxidants in ophthalmic treatments

With the knowledge that there is a natural range of the histidyl dipeptides within mammalian tissues we investigated possible synergism between them in respect to antioxidant activity in ophthalmic treatments. Considering the efficacy of chronic *N*-acetylcarnosine eye drops to the eye in the therapeutic treatment of mature cataracts, we also considered that declining visual acuity, along with the onset of 'halo' effects and vitreous lesions, including floaters and even elevated intra-ocular pressure (IOP), may be a result of diminished GSH levels within the lens and the ocular structures. The oral consumption of the *N*-acetylcysteine ingredient in the oral Can-C Plus ophthalmic formulation boosts the reduced glutathione level in the human crystalline lens.

In a separate pilot study the treatment of a mature human age-related cataract was undertaken over a period of 5 months, with Can-C™ *N*-acetylcarnosine eye drops (two drops twice daily) and oral Can-C Plus capsules (three capsules daily)*, including non-hydrolyzed carnosine, histidine, *N*-acetylcysteine and D-panthethine as the main ingredients /78/. Mature age-related cataract was significantly reversed within 6 months of the combined treatment. Can-C Plus supplement was effective in treatment of senile cataracts as a monotherapy oral product (reviewed in /78,79/). We found that the L-histidine ingredient in the oral Can-C Plus acts synergistically with other natural imidazole-containing peptidomimetics (*N*-acetylcarnosine, non-hydrolyzed L-carnosine) as antioxidants. Our results show that combining imidazole-containing compounds at near physiological concentrations results in heightened synergistic antioxidant and chaperone-like activity. The clinical and experimental data demonstrate the effectiveness and safety of this combined treatment modality /78,79/.

* Details of the formulation are available from Innovative Vision Products, Inc.

4. DISCUSSION

The presented morphological and biochemical studies of human cataracts demonstrate that damage to the lens fiber membrane is involved in the early stages of the development of the opacity. In the course of ageing, in which, however, the lens transparency is still preserved, we may note increasing changes in the regular order of the lens fiber cell packing. We hypothesize that this is related to the detergent action of the phospholipid-oxidation products accumulated in lenticular fiber membranes, which impair lipid-lipid and protein-lipid interactions. In this way changes are induced in membrane geometry /15/. Phospholipid molecules modified with oxygen, incorporating themselves into the lipid bilayer, change its geometry, diminishing the area of its hydrophobic portion, and increasing its surface curvature owing to shortened radius of membrane particles. This effect is obviously the basis of the membrane fragmentation and smaller vesicle formation in cataract. It is also possible that the change of lenticular membrane bilayer geometry can occur under the influence of phospholipid formation with Ca^{2+} ion complexes. An intrusion of proteins from the membrane surface to the water surroundings occurs as a result of impairment of the hydrophobic-hydrophilic balance of the lipid bilayer. Membrane integrity is disrupted during the development of cataract. The mature cataract showed various types of cell disruption in the perimeter but not in the core of the nucleus. In line with another published study of age-related or senile human nuclear cataracts in which morphological changes were examined using electron microscopy of thin sections /80/, our data suggest that these disruptions were globules, vacuoles, multilamellar membranes, and clusters of highly undulating membranes. Other potential scattering centers found throughout the mature cataract nucleus included variations in staining density between adjacent cells, enlarged extracellular spaces between undulating membrane pairs, and protein-like deposits in the extracellular space. Subtle structural changes, especially small fluctuations in protein density between adjacent cells and alterations of the membranes and the extracellular space, probably contribute significantly to the central opacities in human nuclear cataracts /80/. Such observations, considered as a whole, suggest that an understanding of the triggering mechanism of the lens fiber membrane damage is important to elucidate the causes of lens ageing

and human cataract formation. These manifestations are typical for the development of free-radical oxidation reactions in biological membranes /15/.

Kinetic curves of the accumulation of different molecular LPO products in the lens during cataract development correspond to the scheme of their gradual transformation in biological membranes when their auto-oxidation is induced: phospholipids → hydroperoxides of phospholipids → carbonyl compounds (MDA) → intermolecular cross-links of Schiff base type. This sequence of formation of lens phospholipid auto-oxidation products in the course of cataract development provides a demonstration of the lenticular membrane LPO activation being a leading factor in cataractogenesis. The variety of the LPO products, as well as of the processes developing as a result of induction of auto-oxidation in lens fiber cell plasma membranes, determines a large range of peroxidative effects on the opacification of the lens. The most characteristic manifestations can be included in these effects: disintegration of lenticular fiber plasma membranes, formation of new blue fluorophores, and formation of high-molecular aggregates leading to light-diffusion in lenticular matter. Oxidation of membrane lipids could directly or indirectly alter the molecular structure of lens membranes. Development of peroxidative reaction in the lipid membrane phase is accompanied by the accumulation of fluorescent products which have fluorescence excitation and emission spectra parameters similar to those of cataractous fluorophores /81/. In our previous study a tentative investigation of the physico-chemical nature of the fluorophores accumulated in lenticular lipids during cataract development was undertaken /43/.

Protein oxidation in the lens has been shown to initiate at the membrane /6/, and products of lipid peroxidation in the human lens increase with both age and cataract /23/.

In experimental animal models, LPO products have been reported to be a cause of cataract /21,31,81/. Borchman *et al.* found that lipid oxidation is an early event in UVB-induced damage in lens epithelial membranes /82/. Together with polymerization of membrane-linked crystallins, the accumulation of LPO products in lenticular membranes can lead to inhibition of membrane-bound enzymes, impairment of protein-lipid interactions in the membrane, and also of molecular interactions of the membrane with the cytoskeleton and water-soluble lenticular crystallins. LPO products can act as ion transfer inductors in

the lens, increasing Na^+ and Ca^{2+} ion intracellular concentrations at corresponding stages of cataract development, thereby enhancing the opacification progress /83/.

What are the mechanisms of intermolecular protein cross-links formation in cataract? Dialdehyde bifunctional reagents are characteristic of LPO products, and their interaction with free amino groups entails Schiff base formation and, subsequently, inter- and intramolecular cross-links. It should be pointed out that formation of phospholipid intermolecular cross-links was shown in this study by the characteristic fluorescence of lipid extract from cataractous lenses. Covalent cross-linking of lenticular proteins in cataract may also be possible as a result of their interaction with the lipid-free radicals appearing in the course of the LPO process. Cross-linking of biomolecules by the reaction of the carbonyl groups of MDA and amino groups of amino acids, proteins, nucleic acids and their bases, and phospholipids produces lipofuscins, fluorescent Schiff-base conjugates resulting in the formation of high molecular weight aggregates chronically accumulated in cataracts and increased in mature cataracts, as well as inactivating enzymes in the lens /14, 15,43/.

The primary molecular LPO products, i.e., phospholipid hydroperoxides and their *in vivo* indicator, diene conjugates, represent the major change in the lipid composition of the aqueous humor during cataract formation. At the stages of mature cataract, end fluorescent LPO products are distinctly detected. The crystalline lens is well equipped with antioxidant defences showing some specificity in their action. LPO in the lens incubation system was decreased in the presence of free radical scavengers and enzymes that degrade H_2O_2 (EDTA, SOD, catalase, L-carnosine and chelated iron). The most effective agent was EDTA which chelates the free metal cations required to generate $O_2^{-\cdot}$ radicals that initiate the free radical process culminating in LPO. Metal-catalysed oxidation reactions of the lens reductants (GSH, ascorbate, lens crystallins containing SH groups) was proposed as a basic mechanism for release of free radicals or lipid hydroperoxides in the lens medium, and the rates and final levels of oxidant formation by lenses were found inversely dependent on their integral degrees of clouding /50/. Since LPO is clinically important in many of the pathological effects and ageing, new therapeutic modalities should treat the incessant infliction of damage to the lens

cells and biomolecules by reactive lipid peroxides and oxygen species, and 'refashion' the lens membranes affected by the lack of important metabolic 'detoxification' of phospholipid peroxides. L-Carnosine and its ophthalmic prodrug form *N*-acetylcarnosine are part of this group of products /22,52,60/. These compounds act as universal antioxidants with established ability to give efficient protection against LPO both in the lipid phase of cellular membranes and in the aqueous environment, by protecting proteins, DNA, and sugars from oxidative damage. Various protective antioxidant enzymes, such as SOD or catalase, can react with their substrates only in an aqueous environment /84/.

The data of this study demonstrate that when a naturally occurring compound *N*-acetylcarnosine is combined in the ophthalmic formulation Can-C™ with a lubricant carboxymethylcellulose, an increased uptake of L-carnosine in the aqueous humor occurs in topically treated rabbit eyes over the application of pure *N*-acetyl-carnosine compound in the control buffered ophthalmic formulations. The ophthalmic topical application of pure L-carnosine (1% solution) to rabbit eyes (instillation, subconjunctival injection, ultrasound-induced phonophoresis) to produce the desired effect, whether this is therapeutic or prophylactic, did not lead to the intraocular absorption or accumulation in the aqueous humor of this dipeptide compound /60/. Thus the pharmaceutical compounding and formulating of the *N*-acetylcarnosine drug into suitable dosage forms for human and veterinary administration is important according to the developed pharmaceutical formulations /85/ describing a combination of the pharmacologically active *N*-acetylcarnosine with a cellulose com-pound to enhance activity, reduce side effects of pure L-carnosine, and to modify the drug's action in a way to make it more suitable for the treatment of cataracts. Additionally, after absorption into the aqueous humor from the ophthalmic time release *N*-acetylcarnosine carrier, L-carnosine passes into the lens, according to the cited studies, gaining access to prevent and manage the oxidative damage to the lens cells and tissues. In the present study we assessed *in vitro* the uptake of L-carnosine as antioxidant into the crystalline lens during the chronic use of the pharmaceutical drug form required for the patient to self-medicate /85/. Very recently /86/, the ocular pharmacokinetics of carnosine 5% eye drops was evaluated following topical application using the following methods: carnosine 5% eye drops were topically

applied repeatedly (50 μl x 4) at an interval of 5 min. Aqueous humor and lens were collected after 5, 15, 30, 45, 60, 90, 120, 150, and 180 min. Carnosine concentration was determined by high performance liquid chromatography-tandem quadrupole mass spectrometer (HPLC-MS/MS). Carnosine concentration in treated eyes was significantly higher than control eyes. Peak concentration (C_{max}) of carnosine in treated aqueous humor occurred 60 min following topical administration, with the administrated concentration (total endogenous concentration) of 40.9 ± 18.9 μg/ml. Carnosine concentration in treated lens rose to an effective level rapidly and changed slightly with time after topical administration. The concentration of carnosine in the lens at the last time point (180 min, 1.92 ± 1.65 μg/ml) was not significantly different from the highest value (15 min, 2.11 ± 1.83 μg/ml) /86/. These experiments present the rather artificial experimental situation with multiple ocular topical use within a very short period of time (5 min) of the excessive 5% carnosine concentration. The reported data should be considered as the inhibition of tissue carnosinase enzymatic activity in the cornea and conjunctiva of the eye, normally hydrolyzing carnosine under physiological conditions, with substrate (carnosine) overload. The frequent topical administration of excessive concentrations of carnosine can manifest signs of ocular allergy. Ocular physicians should be cautious about the described overload regimen with 5% carnosine in ocular tissues. The visualized effects of 1% *N*-acetylcarnosine with the lubricant carboxymethylcellulose on age-related cataracts in clinical cases have been demonstrated by the author's group /87/. These observations suggest that *N*-acetylcarnosine may prevent cataracts. However, there is also evidence suggesting that *N*-acetylcarnosine reverses lens opacity in humans /62-64/. The mechanism for this observation may involve carnosine's ability to disaggregate glycated α-crystallin protein /88/. In the cataractous lens, cross-linking of proteins by any means increases their effective molecular weight and produces light scattering and consequent lenticular opacity. The production of such high molecular weight protein complexes by disulphide bridges and covalent links with dialdehydes has been implicated in the formation of senile and other cataracts /89/. Both types of cross-linking may be caused by depletion of the lenses' GSH and accumulation of LPO products in the lens tissue. The results of our studies strongly suggest that L-carnosine released from its ophthalmic prodrug *N*-acetylcarnosine during its

topical application to eyes with cataracts is able to prevent the loss of GSH and to remove the secondary LPO products in biological systems /21/. This, in turn, may lead to dissociation of the intermolecular protein cross-links due to a glutathione-protein thiol-disulphide exchange mechanism and utilization of lipid peroxides and dialdehydes derived from the LPO process, anchoring protein-lipid complexes in the lens /21/. A possibility exists from our studies that carnosine reacts directly with MDA and other aldehydes/ketones. Indeed, carnosine has been shown to protect against MDA-induced crosslinking and toxicity, and a hydroxynonenal-carnosine adduct has recently been characterized, providing further evidence for carnosine's potential as an aldehyde scavenger /90/. The presented results can be explained in part by the adduction of the various LPO products directly by carnosine following de-acetylation of *N*-acetylcarnosine. The published results suggest that histidine is the representative structure of L-carnosine for an anti-crosslinking agent, containing the necessary functional groups for optimal protection against crosslinking agents /91/.

LPO reactions are widely involved in the genesis of ophthalmic disorders, such as cataract, glaucoma, and inflammatory, corneal, retinal, and systemic disorders having a component of oxidative stress in their genesis /22/. These are the most widely used application sites for drugs and pharmaceutical products besides ophthalmic surgery. However, products are usually swallowed in order that the active ingredient can be absorbed from the gut and gain access to the blood stream and, in this way, secondarily to the eye. While some parts of the eye are richly supplied with blood vessels, others including the crystalline lens are not. Moreover, the response of systemic drugs and oral supplements can be variable, as other gut contents can influence the absorption of alimentary-administered remedies, and in particular, being mostly peptidergic products, they are susceptible to proteolytic breakdown by proteases, dipeptidases, etc., encountered during internal digestion in the gastrointestinal tract or transport in the blood stream, and consequently have a too limited half-life upon systemic application. The present study presents pharmacokinetic evidence suggesting that the developed ophthalmic prodrug *N*-acetylcarnosine time-release form (Can-C^{TM}) is effective in making L-carnosine bioavailable for intraocular absorption into the aqueous humor. This is by far the most effective route of administration of L-carnosine for the

eye and forwarding it to the lens tissues. Topically applied ophthalmic formulation produces effective levels of L-carnosine in the anterior segment of the eye. The response of the patient to the drug will to a great extend depend on the concentration of L-carnosine that is available at the site of action near the lens in the aqueous humor. The relationship between the dose or concentration administered and the final L-carnosine concentration at the focus of action near the lens in the anterior segment of the eye is the resultant of normal pharmacokinetic processes of *N*-acetylcarnosine deacetylation which are themselves subject to the effect of other agents and excipients (such as carboxymethylcellulose, vitamins, buffers, preservatives, pH adjusters) /85/. Our studies document the optimal bioavailability of an ophthalmic formulation including 1% *N*-acetylcarnosine with a lubricant, carboxymethylcellulose, and predict further benefits of the application of this drug in prevention and reversal of age-related cataracts in human and animal eyes /85,87/.

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