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Lipid fluorophores of the human crystalline lens with cataract

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Abstract. It has been established that the development of cataract is accompanied by the formation of various fluorophores in the lipid fraction of the lens. These lipid-fluorescing products have been separated chromatographically according to polarity and molecular weight. It is shown that the initial stages of the development of cataract are characterized by the appearance of lipid fluorophores in the near ultraviolet and violet regions of the spectrum (excitation maximum 302–330 nm, emission maximum 411 nm) with low polarity and a small molecular weight; the maturing of the cataract is characterized by an increase in the intensity of the long-wave fluorescence of the lipids in the blue-green region (430–480 nm) and by the formation of polymeric high-molecular-weight fluorescing lipid products with high polarity. It has been demonstrated that the appearance of lipid fluorophores in the crystalline lens is associated with the free radical oxidative modification of the phospholipids and fatty acids in cataract.

Introduction

Two most characteristic manifestations of cataract exist at the molecular level – the formation of high-molecular-weight protein aggregates and the appearance of "azure" fluorescence of a nontryptophane nature [17, 29].

Among the post-translational changes in cataracts found in human lens protein is a yellowing [4] and fluorescence originating from compounds other than normal aromatic amino acids (atypical fluorescence) [1, 21, 31]. The formation of high-molecular-weight protein aggregates is thought to be the cause for the lens opacification [29], while the photochemically induced chromophores seem to be responsible for the coloration [35]. The fluorescence intensity of some of these chromophores has been determined at two wave lengths (440 and 520 nm) after excitation with a broad, long wavelength band of ambient UV radiation (300–400 nm) using a modified Scheimpflug slit lamp camera (UV slit lamp densitography) [18]. It could be shown that the lens fluorescence increases with age and particularly in human cataract formation. There is an abnormal fluorescence caused by occupational exposure to UV radiation (>300 nm) or in patients on chronic PUVA (psoralen UVA) therapy.

Despite intensive studies, not much is known about either the chemical nature of these chromophores or the molecular mechanism(s) of cataract formation. In order to explain the appearance of these unusual fluorescent compounds in the lens, a number of mechanisms have been postulated, including: (a) direct oxidation with hydrogen peroxide [30, 34]; (b) direct photolysis [11, 15, 22, 38]; (c) photosensitized oxidation [5, 35, 37]; (d) reactions with glucose [25]; (e) reactions with ascorbic acid [6, 23]. However, a lipid component of the blue-green fluorescent products formed in the lens has not been studied.

Recently it was shown that, together with protein aggregation as a result of the formation of intermolecular disulfide bonds [29], the high-molecular-weight aggregates also include nondisulfide (not sensitive to the action of reductants of the S-S bonds) associates of covalently cross-linked membrane proteins [29]. In this connection we would note that one of the widespread mechanisms for the formation of conglomerates of membrane proteins not "uncross-linked" by reductants and stable in the presence of detergents is the process of lipid peroxidation (LPO) [33]. LPO is implicated in many pathological events including aging, coronary heart diseases, and cancer [32]. We have recently revealed lipid peroxidative mechanism involvment in cataractogenesis [3]. Together with the formation of high-molecular-weight protein aggregates the development of the reactions of peroxidation in the lipid phase of the membranes is accompanied by the accumulation of fluorescing products having the parameters of the excitation and fluorescence emission spectra close to those for cataract fluorophores [12, 18]. This prompted a hypothesis on the participation of LPO reactions in the pathogenesis of cataract [3].

Previously we showed that with the development in man of cataract in the lipids of the crystalline lens various molecular LPO products accumulate, including those with characteristic fluorescence. This fluorescence of substances of a lipid nature ($\lambda_{fl}^{max}=435$ nm, $\lambda_{excit}=365$ nm) may point to the formation of Schiff bases in the lenses in cataract [33]. In fact, recently there was evidence to suggest that Schiff-base conjugate can be formed in human cataracts [8]. Bhuyan et al. [8] demonstrated a presence of phospholipid-malondialdehyde adduct (the condensation product of malonic dialdehyde with the amino groups of the phospholipids and proteins fluorescing in the 420–460 nm region) in senile human cataract. But the presence of several fluorophores in the human cataract lens and the complex form of the fluorescence excitation spectra of the lens [19] cannot be explained by the accumulation in the cells of only Schiff bases. In this study we tried to investigate the physical

chemical nature of the fluorophores accumulating in the lens lipids during development of cataract.

Materials and methods

Subjects

In our work we used clouded human lenses – mature senile and immature complicated cataracts ($n=17$) obtained at surgery by the method of intracapsular cryoextraction. Transparent lenses were extracted from freshly donated eyes ($n=19$) obtained from the eye bank. Before surgery all the lenses were studied by biomicroscopy and assigned to mature cataract or transparent lenses in line with the clinical characteristic of clouding. The average age of cataract patients and donors was 65 ± 9 years.

Lipid extraction procedure

Immediately after the material had been obtained, lipids were extracted by the Folch method [16]. The extraction was carried out by tissue homogenization in 20 volumes of chloroform-methanol mixture (2:1) by volume with 4-methyl-2,6-ditert-butyl-phenol 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 in a 7:1 ratio to promote the stratification. A temperature of 0° C was maintained for all the operations. After the separation of the phases and removal of the aqueous-methanol layer, the lower chloroform fraction was evaporated. The phospholipid content was assessed according to the results of organic phosphorus evaluation [36]. Total amount of lipids in the extract was determined gravimetrically, as well as by characteristic absorption in a 206- to 210-nm area of the lipid sample after dissolution in 4 ml of a methanol-heptane mixture (5:1 by volume).

Detection of lipid peroxidation products

Accumulation of LPO primary 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 products absorbency at 274 nm, corresponding to the concentration of conjugated trienes and cetodienes [10] on a "Hitachi-557" spectrophotometer (Japan). The content of the end LPO products with characteristic fluorescent parameters was determined from the lipid extract fluorescence intensity at 365-nm excitation and 420- to 440-nm emission wavelengths [12], measured on a "Hitachi-MPF-4" spectrofluorometer. The source material content in the samples was equalized by varying amounts of phospholipids. The spectrofluorometer was calibrated at the beginning of every working day against a solution of quinine sulfate (1 μM in 0.1 N H_2SO_4) standard, at 435-nm fluorescence emission and 365-nm excitation wavelengths.

Chromatographic separation of lipids

To demonstrate the lipid nature of the fluorescence detected by us, we fractionated the chloroform-methanol extracts of the lenses in a column of Sephadex LH-20 followed by

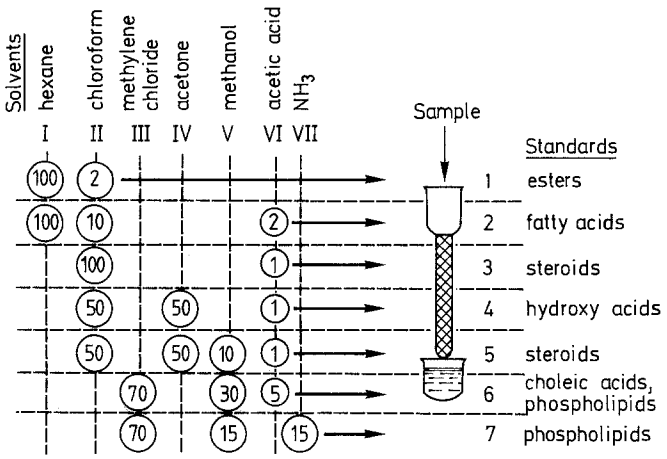


Fig. 1. Microcolumn liquid chromatography. The lipid sample is applied to a glass column filled with silica gel (after extraction and concentration). The eluates are collected as pools of the fractions of the automatic specimen processor. The components (and their ratios) of the mixtures of the fractions eluted from the columns are presented schematically: I-hexane; II-chloroform; III-methylene chloride; IV-acetone; V-methanol; VI-acetic acid; VII-NH_3. The fractions where the standards concentrate are applied to the columns: 1-esters; 2-fatty acids; 3-steroids; 4-hydroxyacids; 5-steroids; 6-cholic acids, phospholipids; 7-phospholipids

quantitative recording of the fluorescence of the eluted fractions by the Csallany method [13]. The column was calibrated by applying a lipid extract from an adult rat brain. This was a control for the aged material used in our study. To separate the lipids by polarity we used microcolumn liquid chromatography with a PREP-1 automatic specimen processor (Du Pont Instruments, USA) with a 12-column rotor and modified glass columns (3×66 nm) filled with Rsil PREP Silica silica gel (Alltech Associates, USA) with a particle diameter of 25–40 μm.

The chromatographic method is given in Fig. 1. The system of fractionation by mixtures of solvents with increasing polarity possesses raised selectivity in relation to substances with different functional groups. Based on the earlier calibration, and applying a series of standards, it was established that the fatty acids are eluted in the second, cholesterol in the third, phosphatidyl choline in the seventh, and phosphatidyl ethanolamine in the sixth and seventh fractions. Oxidation of model samples of the lipids was by threefold insonation with a Lab Line Ultratip Labsonic System ultrasonic disintegrator with 120 W power for 7 min at room temperature. In our study we employed arachidonic and linoleic acids and phosphatidyl choline and phosphatidyl ethanolamine from Serva.

^{1}H-NMR spectra

After chromatographic separation, lipid fluorescent products were dissolved in $CDCl_3$ and proton magnetic resonance (PMR) spectra were carried out on a Varian FT-80 Spectrometer (Varian Associated, Palo Alto, CA, USA), operating at 80 MHz for ^{1}H at 20° C using 10-mm sample tubes. The chemical shifts (δ), digitally computed with an error of ±0.005 ppm, are relative to internal tetramethylsilane (TMS), obtained from Merck (Darmstadt, FRG), as a reference compound. The ^{1}H-spectra were obtained with Fourier transformation of an average of 64 free induction

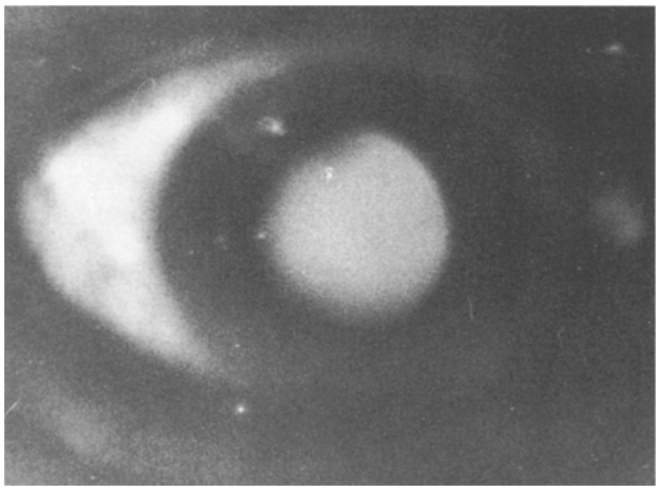


Fig. 2. Blue-green fluorescence of the lens, registered in the eye of a patient with mature cataract. The intensity and the spectral band of the lens fluorescence correlate with the parameters of extracted lipid fraction

decays (FID), except for the ultrasound-induced auto-oxidized PUFA, which was averaged over 1000 scans. Mass spectroscopy of the fractions was carried out on a Ribermag R-10-10-C instrument (Wermag, France).

Effect of $NaBH_4$ reduction on the fluorescence intensity

In a screw-cap (Teflon) culture tube, 10–20 mg of the cataract lens nucleous tissue were dissolved in 5.0 ml water. NaOH (5.0 ml of a 0.2 *M* solution) was added and then 100 mg of $NaBH_4$. The tube was flushed with N_2 and capped. The reaction was allowed to proceed at 45° C for 22 h. At the end of the incubation period 1.0 ml of 4 *N* HCl was added and the sample was lyophilized. Then a lipid extraction procedure was performed. Chromatographic separation of lipid extracts was done as described above. Fluorescent spectra of chromatographic lipid fractions were obtained on a spectrofluorometer. In experiments where lens tissue was reduced with $NaBH_4$ after reduction, salts were removed by dialysis prior to the lipid extraction procedure. Background absorption and fluorescence contributed by the non-lens lipid constituents were subtracted.

Clinical evaluation of the lens luminescence

Lens autoluminescence was registrated directly in the patient's eye utilizing a SVD-120 A (USSR) mercury lamp as the excitation light source. Quantitative assessment of the fluorescence intensity at 330- to 400-nm excitation and 430- to 460-nm emission wavelengths was performed by selection of corresponding light filters, using photographic registration on KODAK CF 1000 film of the eye with consequent scanning of the negative.

Results

Detection of lens fluorescent products

Figure 2 shows the characteristic blue-green fluorescence of the lens which we managed to register directly from the cataractous (mature cataract) eye of a patient. We have characterized at least two types of chromophores in the human cataractous lens; one absorbs at approximately 335 nm and fluoresces at 405 nm and the second absorbs at approximately 365 nm and fluoresces at 440 nm (Figs. 3, 4b). Using various concentrations of phosphorus, it was confirmed (Fig. 3b) that the height of the fluorescent peaks was directly proportional to the concentration of phospholipids in chloroform extract.

It turned out that the lens fluorescence excitation spectra have a complex pattern, while the maxima position coincides with that on the excitation spectra of the fluorophores accumulated in the lipid fraction of the same lens (Fig. 3a). Table 1 summarizes the results of lipid fluorescence measurements on normal and cataractous human lenses of variously aged patients. Two fluorescent compounds increase in concentration in the lipid phase as the lens ages, and there is a more progressive increase in the fluorescence in-

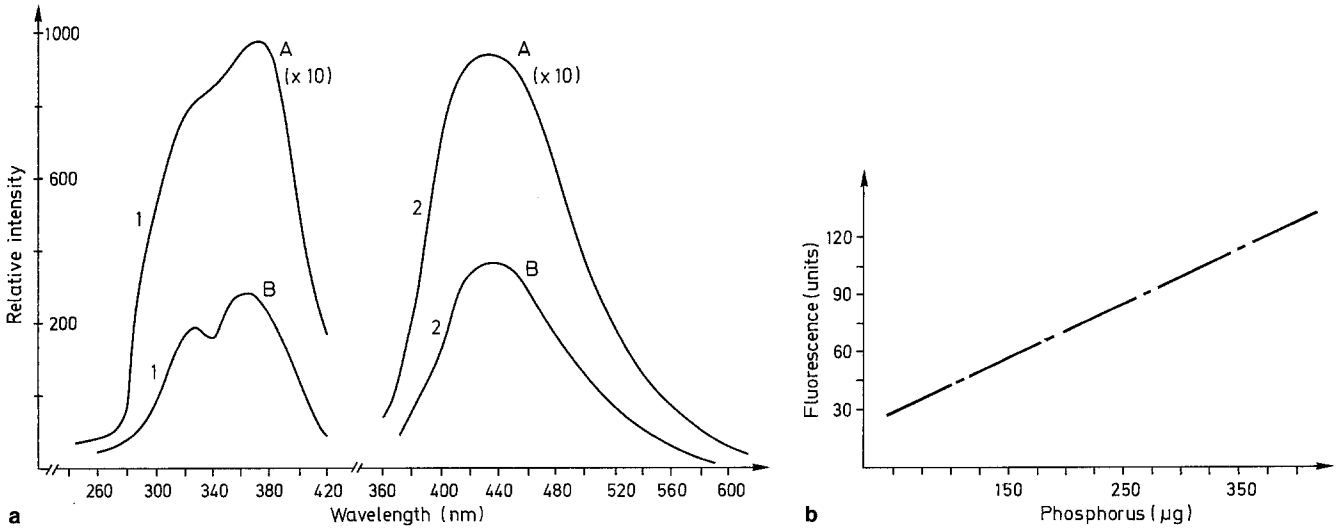
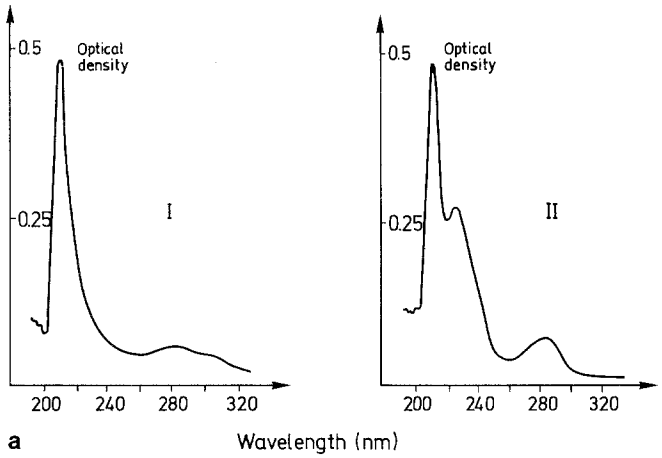


Fig. 3. a Fluorescence spectra characteristic of cataract. *A*, Mature cataract, a whole lens; *B*, lenticular lipid extract from the same lens; *1*, excitation spectra at emission 438 nm; *2*, fluorescence emission spectra on excitation 365 nm. **b** Relationship between concentration of phospholipids and corresponding fluorescence intensity of chloroform extracts from mature human cataracts. The relative fluorescence of quinine sulfate (1 µM in 0.1 *N* H_2SO_4) standard was 1200 units at emission 438 nm on excitation at 365 nm



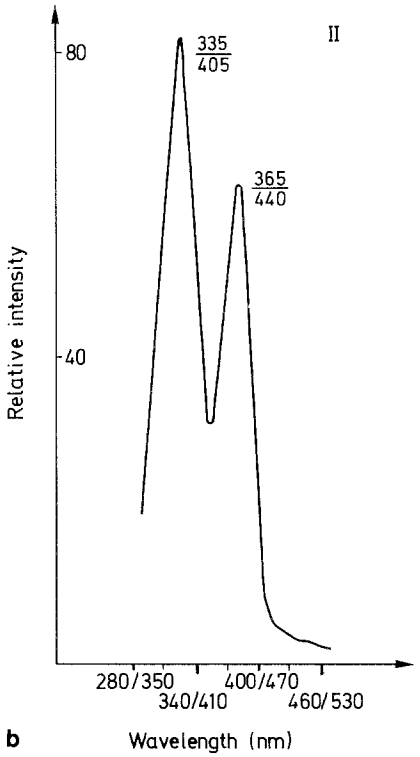


Fig. 4a, b. Characteristic ultraviolet absorption (**a**) spectra (*I*, transparent lens; *II*, immature cortical cataract) and fluorescence (**b**) (doubled monochromators) of lipid extracts in the 5:1 methanol + heptane mixture. Both lenses were obtained from persons 65 years old. Registered fluorescence maxima were at wavelengths of excitation and emission 335/405 nm and 365/440 nm respectively

tensity at emission 438 nm on excitation at 365 nm with the development of cataract.

Figure 4a presents typical UV absorption spectra of the lipid extracts from transparent and cataractous lenses. The maximum in the 206 nm regions corresponds to absorption of isolated double bonds of hydrocarbon chains. In absorption spectra of lipid extracts from lenses with cataract there were two additional maxima, at 232 and 274 nm. The first of them corresponds to absorbence of diene conjugated structures. The maximum at 274 nm corresponds to triene conjugates. The maximum at 232 nm was virtually absent in lipids extracted from transparent lenses, and the maximum at 274 nm had significantly smaller amplitude than

Table 1. Lipid fluorescence of normal and cataractous human lenses

Lens description	Number of lenses	Fluorescence[a] (units/mg lipid)
Normal, 40 years old	5	24.6± 3.2[b]
Normal, 60 years old	8	30.1± 3.6
Normal, 70–80 years old	6	36.7± 8.1
Immature cataract, 60–80 years old	4	90.2±16.2[c]
Mature cataract, 60–80 years old	13	195.6±22.0[d]

[a] The relative fluorescence of quinine sulfate (1 μM in 0.1 *N* H_2SO_4) standard was 1200 units at emission 438 nm on excitation at 365 nm
[b] Mean±S.D.
[c] $P<0.02$ compared with normal lenses of the same age-group
[d] $P<0.001$ compared with normal lenses of the same age-group

in lipids from opaque lenses of persons of the same age. It was found that the content of lipid products having conjugated double bonds and determined by characteristic maximum in a UV spectrum at 232 nm increases at the initial stages of the lens opacification. However, at the further stage of mature cataract the level of these products falls. At the same time, determination of the lipid fluorescent (365/438 nm) product concentration in the lenses revealed their prolonged accumulation with the rise in degree of opacity.

Chromatographic and spectral studies of lipid extracts

On chromatography of the lipid extracts of the cataract and normal-aged lenses in the Sephadex LH-20 column the fluorescing products separated into two distinct fractions differing in the relative intensity of their fluorescence ($\lambda_{em}=$ 438 nm, $\lambda_{excit}=365$ nm) (Fig. 6b). Ageing is accompanied by the accumulation in the lipid lens fraction of a fluorophore with low molecular weight ($M<400$), which is eluted from the column by the eluent volumes (33–37 ml). Cataract development is accompanied by the formation in the lens of a lipid fluorophore with high molecular weight ($M>$ 1000) which is eluted from the column in the first 15 ml of the eluent and also low-molecular-weight ($M<400$) fluorescent products eluted from the column with far larger eluent volumes (32–38 ml).

The absorption spectrum of the high-molecular-weight fraction (Fig. 5b) is characterized by an even drop in optical density in the region of 220–280 nm and by the absence of maxima at 232 and 274 nm characteristic of the initial LPO products. The absorption spectrum of the fluorescing low-molecular-weight fraction has two marked maxima in the 232- and 274-nm region corresponding to absorption in this spectral region of the initial lipid extracts (Figs. 4a, 5b). Fluorescence excitation spectra of light fractions demonstrated two maxima, in the 365-nm region and in the 302- to 330-nm region. On chromatographic separation of the lipid fluorescent products by microcolumn liquid chromatography in a system allowing one to fractionate substances of a lipid nature by polarity (Fig. 1), a high correlation was noted between the presence of the maxima in the ultraviolet absorption spectra of the initial lipid extract of the lens in the 232- and 274-nm region and a third fraction with weak polarity eluted from the column (Fig. 5a). The formation of such products close in polarity to cholesterol

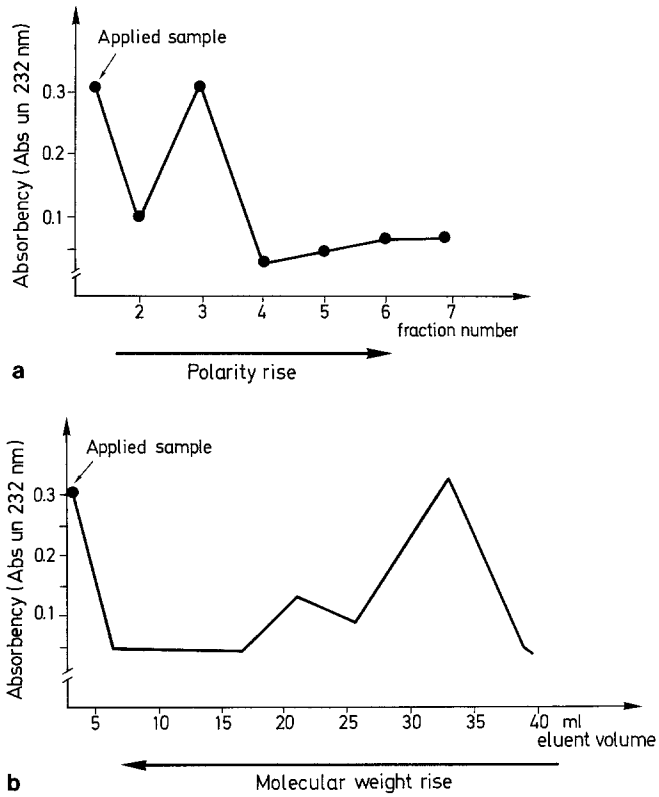


Fig. 5a, b. Chromatographic profiles of the lens lipids eluting: **a** separation by polarity of lipid peroxidation products, absorbing at 232 nm; **b** separation by molecular weight of lipid peroxidation products, absorbing at 232 nm

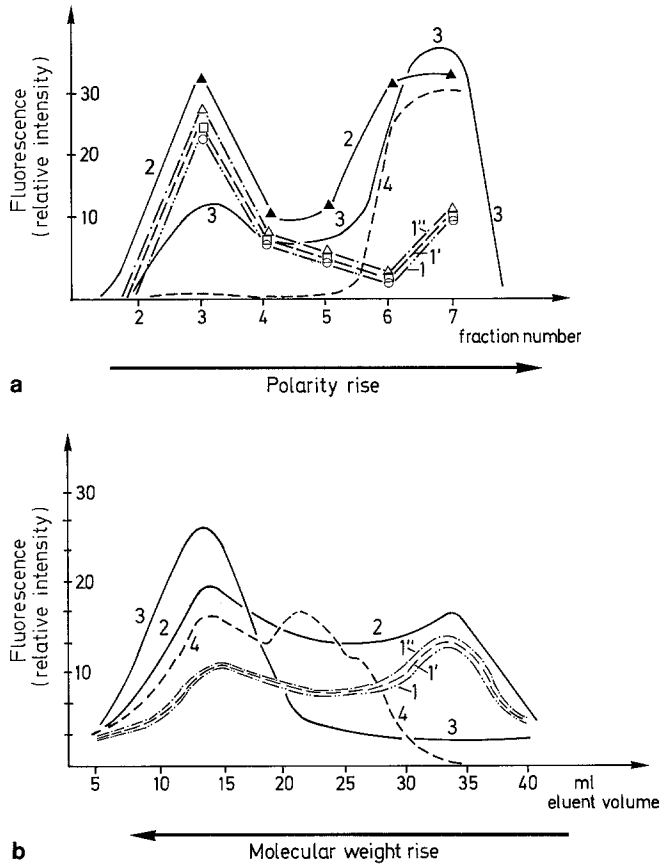


Fig. 6a, b. Chromatographic profiles of the lens lipids eluting: **a** separation by polarity of lipid-fluorescing products – *1*, 40-year-old normal human lens; *1′*, 60-year-old normal human lens; *1″*, 75-year-old normal human lens; *2*, cataractous human lens (mature cataract), 60 years old; *3*, lipid fluorescing products from the adult rat brain; *4*, distribution of phosphorus in chromatographic fractions; **b** separation by molecular weight of lipid fluorescing products – *1*, 40-year-old normal human lens; *1′*, 60-year-old normal human lens; *1″*, 75-year-old normal human lens; *2*, cataractous human lens (mature cataract), 60 years old; *3*, lipid-fluorescing products from the adult rat brain; *4*, curve of distribution of phosphorus in chromatographic fractions

apparently reflects the appearance of a hydroperoxide group in the fatty acid molecule simultaneous with the formation of the diene conjugated structure changing the absorption spectrum in the 232-nm region.

On recording the fluorescence-excitation spectra of the third fraction we noted the presence of two maxima, in the 365- and 302- to 330-nm regions. The intensity of the second maximum is greatest at the emission wavelength of 411 nm. The fluorescing products of oxidation of the lens lipids in cataract, absent in the controls, were present, in the main, in the sixth and seventh lipid fractions eluted from the silica gel columns (Fig. 6a). The fluorescence maximum shifted to the longer wave region of 430–460 nm with predominance in the excitation spectrum of the maximum in the 365-nm region. The products of this long-wave fluorescence responsible for the most reliable differences between the mature cataract and transparent lenses are characterized by the greatest polarity as compared with the other fractions. The parameters of the absorption and fluorescence spectra of these fractions repeat the form of the spectra of the high-molecular-weight fluorescing lipid fraction. The third fraction of separation by polarity has spectral characteristics similar to those of the low-molecular-weight fluorescing fraction (Fig. 5a, b). Lipid fluorophores extracted from normal aged lenses were localized in the third fraction (Fig. 6a).

The atypical lipid fluorescence intensity decreases after reduction with $NaBH_4$. This may confirm the Schiff-base type of high-molecular-weight fluorescent products. A similar effect takes place in the sixth and seventh fractions with high polarity.

To confirm the lipid and phospholipid nature of fluorescing pigments in cataractous lenses we determined the phosphorus content in the chromatographic fractions (Fig. 6a, b). We also calibrated Sephadex LH-20 and silica gel columns by application of an adult rat brain lipid material as a control lipofuscin-linked sample. As seen in Fig. 6a, b, the phosphorus-containing elution profile has a maximum corresponding to high-molecular-weight fluorescing fractions. Aged material obtained from transparent human lenses eluted by volumes differed from lipofuscin-like fluorescing pigments isolated from the adult rat brain (Fig. 6a, b), but the mentioned lipid component of lipofuscin-like rat pigments corresponded precisely to the fluorescing high-molecular-weight and polar fractions accumulated in mature human cataract. These facts reflect the idea that two distinct types of lipofuscin-like pigments are accumulated in aged and cataractous human crystalline lenses. What is the nature of the fluorochromes accumulated in low-molecular-weight fractions with low polarity, more typical for lipids extracted from aged normal and early cataracts? As shown in elution profiles of the lens chloroform-

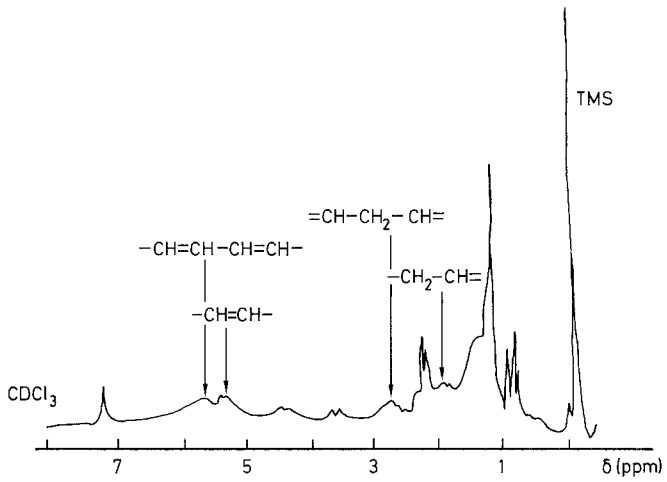


Fig. 7. PMR spectrum of low-molecular-weight fluorescent chromatographic fractions with low polarity of lipids extracted from immature cataractous human lens. The signal attributions (*ppm*) are relative to internal standard tetramethylsilane (*TMS*) as a reference compound. PMR spectra were performed as described under "Materials and methods"

methanol extracts (Fig. 6a, b), these fractions do not contain phospholipids (phosphorus). To state the chemical nature of indicated products we registered ^{1}H-NMR spectra of high settlement and obtained mass spectra of the low-molecular-weight fractions and of the chromatographic fractions with low polarity (Fig. 7). The assignment of the NMR signals was made on the basis of a previous study [28]. Compared with normal lenses (40-year-old age group) a downfield shift of the signal attributable to the fragment C=C–C=C ($\delta = 5.8$) was observed in fluorescent chromatographic fractions. At the same time the resonance due to the $-CH_2-CH=$, $-CH=CH-$, and $=CH-CH_2-CH=$, although present at the same position ($\delta = 1.9$; 5.35 and 2.80, respectively) showed a much weaker intensity. It is evident that in the cataractous chromatographic fractions there is a mixture of different products. In fact, the PMR spectra showed the presence of the unoxidized PUFA molecule and those of the product(s) derived from the oxidation processes. The low-molecular-weight fraction of low polarity showed the presence of nonpolymeric oxidation products of free and long-chain polyenic fatty acids with molecular weight as ($C_{22}H_{32}OO$, i.e., $C_{22:6}$) $328 + 2 \times 32$ ($2O_2$). Binding of two oxygen atoms gives a molecular weight similar to those of maximal molecular ions in the chromatographic fraction. ^{1}H-NMR spectra of a high-molecular-weight lipid fraction showed proton signals of the similar aliphatic lipid groups but differed in ratio. No proton signals of aromatic amino acids were detected. Extrapolation from the data taken as a whole should lead to the conclusion that the fluorescing compounds detected in chloroform-methanol extracts obtained from human cataracts are of a lipid nature.

To study the properties of two types of lipid-fluorescing products accumulated in the lens during aging and cataract development, we induced the oxidation of model lipid samples by insonation and chromatographic separation. The samples of arachidonic and linoleic acids, phosphatidyl choline, and phosphatidyl ethanolamine were studied before and after insonation in the same systems and conditions in which the lipid extracts of the lenses had been fractionated. After oxidation and application to the Sephadex LH-20 column of extracts of these compounds we noted the appearance of a high-molecular-weight fraction ($M > 1000$) fluorescing lipid product. The electron absorption spectrum of these "heavy" fluorescing fractions of the fatty acids and phospholipids showed an even drop in absorption in the 220- to 380-nm region. According to Merzlyak and Kovrizhnih and to Orlov et al. [24, 27], the increase in the intensity of long-wave (430–460 nm) fluorescence in the "heavy" fractions of oxidized phospholipids and fatty acids is most probably linked with the formation of polymerized oxidation products of the lipids. These products result from deep oxidation of the lipids and possess strong polarity. However, in some cases we also detected the presence of a low-molecular-weight fluorescing fraction ($M < 400$) most characteristic of the oxidation products of arachidonic acid and phosphatidyl choline. The absorption spectra of this fraction had characteristic maxima corresponding to the absorption of the conjugated double bonds. On separation by polarity, as in the case of fractionation of the lipid extracts of the lenses, the main differences in the absorption spectra were noted for the third fraction. Differences in fluorescence for the oxidized and nonoxidized lipids were also observed for the most polar fractions – the sixth and seventh.

Bound to the most oxidized polar fractions were the products of long-wave ($\lambda_{excit} = 365$–370 nm, $\lambda_{emis} = 430$–460 nm) fluorescence. After insonation of arachidonic acid, the optical density of absorption for the third fraction at 232 and 274 nm fell heavily as compared with the initial extract, but the fall in optical density was simultaneously accompanied by an increase in the intensity of the fluorescence (410–440 nm) of the third fraction of arachidonic acid. The fluorescence-excitation spectrum of the third fraction was of a complex character, with a marked second maximum in the 302- to 330-nm zone. The results indicate the resolution in the course of oxidation by ultrasound of the primary and the formation of further fluorescing products of arachidonic acid. The fluorescence of phosphatidyl choline in the course of oxidation differed insignificantly from that observed for the fluorescing products of the oxidation of the fatty acids. Oxidation of phosphatidyl ethanolamine was accompanied by long-wave luminescence maxima (438 nm), sharply marked as compared with phosphatidyl choline and correlated with the fluorescence of the Schiff bases.

Discussion

A number of studies from several laboratories have demonstrated the accumulation of fluorescent compounds in the lipid extracts from ageing and diseased tissues [9, 12, 14, 20, 26]. Recent studies have proposed the possibility of LPO products accumulating in the lens during cataract development [2, 3, 7]. It seems that the fluorochrome formed by the reaction between the carbonyl group of malondialdehyde and the primary amino groups of phospholipids consequently participates in the generation of complex yellow-to-brown chromophoric lipoid materials called "chromolipids" or "lipofuscins". It is feasible that the human lens may eventually accumulate significant amounts of the lipofuscins, such that they may act adjunctively with protein chromophores toward lens pigmentation. Studies were therefore undertaken to determine the lipofuscin content

of the human lenses in our work. Earlier, a Schiff-base conjugate was isolated in senile human cataract as a novel spot in TLC which, on quantification, was found to be significantly higher than that contained from the similar age-matched control lenses [8]. Evidence of amino phospholipid adduct was also seen in normal human lenses, although significantly lower in concentration as compared with the cataractous lenses.

There are some difficulties in determining the atypical fluorescence origin, however, because a number of compounds have their fluorescent parameters in the same blue-green band of wavelengths. To demonstrate the lipid nature of the fluorescence detected by us, we fractionated the chloroform-methanol extracts of the lenses using two chromatographic systems of lipid separation, the Sephadex LH-20 column (separation by molecular weight) and microcolumn liquid chromatography system with silica gel-filled columns. Calibration, applying a series of lipid standards, established the precise localization of the cataractous human lens fluorophores extracted by the Folch method in the same chromatographic fractions as the peroxidized samples of fatty acids and phospholipids. Lipid compounds with excitation and emission fluorescent maxima at 350–370 nm and 420–440 nm respectively were eluted in small volumes with Sephadex LH-20 column chromatography and showed thin layer mobility similar to that of polar lipids [13, 24, 27]. The imine bonds thus formed in the cataractous lens were reduced with $NaBH_4$. Since such reduction diminished the atypical fluorescence substantially, one can conclude that malondialdehyde adducts which are fluorescent and contain $NaBH_4$-reducible enamine bonds might contribute greatly to the age-related atypical fluorescence seen in human cataract.

Based on the above data and on the resemblance to liposoluble fractions of animal lipofuscin pigments (lipids from the adult rat brain), the role of LPO in the fluorescent compound formation in the lens has been studied. Thus, in the initial stages of cataract development, substances appear in the lipid fraction of the lens that change the UV absorption spectrum of the lipids. In ageing, and particularly in the event of mature cataract development, there is an accumulation of lipid fluorophores in the lens tissue. The same types of products are formed in the lipid model samples after ultrasound-induced auto-oxidation. These LPO products were concentrated in the same chromatographic fractions as the substances responsible for changes in the absorption and fluorescence spectra of the lipids extracted from crystalline lenses. This fact is evidence of the accumulation of free radical oxidation products of the lipids in human lenses affected with cataract. The nature of fluorescent products in low-molecular-weight lipid fractions with low polarity accumulated in aged lenses and initial cataracts evidently resulted from oxidative transformation of polyene fatty acids undergoing no polymerization. Their formation probably takes place with the involvement of endogenous phospholipase A_2, which is able to attack both nonoxidized and oxidized phospholipids.

The maturation of cataract is characterized by an increase in the intensity of the long-wave fluorescence of the lipids in the blue-green (430–480 nm) region and by the formation of high-molecular-weight fluorescing LPO products with high polarity. These products of deeper oxidation of the lipids are of a polymeric nature. Evidently, they result from the radical polymerization of the phospholipids and fatty acids, i.e., lipofuscin-like pigments. Their concentration is low (not detected in the absorption spectra in the 320- to 370-nm region). Modification of the phospholipid molecules in cataract leads to the appearance of extremely polar products in the fatty acids, which is inevitably reflected in their hydrophilic-hydrophobic balance. Such modified molecules acquire the properties of detergents similar to lysophospholipids. As a result, the protein-lipid and lipid-lipid interactions are disturbed in the membranes of the lens, with fragmentation and formation of small vesicles. Thus, the oxidation of the lipids is a process in the development of cataract just as characteristic as the oxidation of the proteins. It is possible that LPO of plasma membranes might cause conformational changes in membrane proteins, exposing the reactive cataract fluorophores. Regarding LPO as the initiator mechanism of the formation of protein polymers in cataract, one may expect that the use of anti-oxidants will lead to the prevention of cataract development.

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