CHEMICAL NATURE OF FLUORESCENT PRODUCTS ACCUMULATING IN LIPIDS FROM THE LENSES OF MICE WITH HEREDITARY CATARACT

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The most characterisitc manifestation of cataract at the biochemical level are the formation of high-molecular-weight protein aggregates and the appearance of a "blue" fluorescence of nontryptophan nature [4]. It has recently been shown that, besides protein aggregates formed by disulfide bonds [12], other aggregates also are found, which also contain nondisulfide associates of covalently cross-linked membrane proteins [10]. It will be recalled that a widespread mechanism of formation of membrane protein conglomerates, not uncoupled by reducing agents and resistant to detergents, is lipid peroxidation (LPO) [3, 8]. Besides the formation of high-molecular-weight protein aggregates, the development of peroxidation in the lipid phase of membranes is accompanied also by accumulation of fluorescent products, whose spectra of excitation and fluorescence emission are similar in their parameters to those of the fluorophores of cataract [5]. This served as the basis for the hypothesis that LPO reactions are involved in the pathogenesis of cataract [1]. The authors showed previously that various LPO products, including those with characteristic fluorescence, accumulate in lipids during the development of cataract in man. It was important to determine to what extent activation of LPO and the formation of fluorescent products of lipid nature are a universal feature of cataract development.

With this aim it was decided to measure the concentration of LPO products in extracts of lenses from mice with hereditary cataract, homozygous for the dominant cataract gene (the Cat^{Fr} gene). The earliest sign of the action of the mutant gene in Cat^{Fr}/Cat^{Fr} mice is the appearance of ultrastructural changes in the nuclear membrane of the primary lens fibers on the 12th day of embryonic development [2]. Pycnosis of the nuclei is observed in the central zone of the lens in 14-day-old mutant embryos, and at the age of 3-4 weeks this leads to lysis of the fibers with the formation of a nuclear cataract in all the animals [13]. An attempt was made at the same time to study the chemical nature of the fluorophores accumulating in lipids of the lens during cataract development.

EXPERIMENTAL METHOD

Lipids were isolated from mouse lenses by the method in [7]. Chloroform-methanol extracts were fractionated on a column with Sephadex LH-20 (35 × 1.8), calibrated beforehand with lipofuscin and α-tocopherol, and the fluorescence in the fractions was measured on a fluorometer (Hitachi MPF-4, Japan). NMR spectra were recorded on a WUH-360 instrument (Bruker, West Germany) in deuterochloroform and mass spectroscopy of the fractions was carried out on a Ribermag R-10-10-C instrument (Wermag, France). The optical density of the fractions was measured on a spectrophotometer (Specord, East Germany).

EXPERIMENTAL RESULTS

Changes characteristic of a nuclear cataract were observed in the lenses of 30-day-old Cat^{Fr}/Cat^{Fr} mice. In particular, fibers in the central zone of the lens showed degeneration and lysis. The deep cortical layers were swollen and vacuolated, and darkly stained granules

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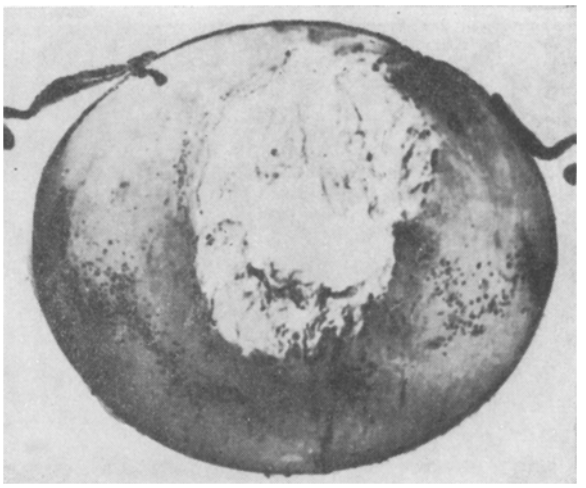

Fig. 1. Sections through lens of 30-day-old Cat^{Fr}/Cat^{Fr} mouse. Extensive area of destruction of lens fibers visible in central zone (cataract masses). Hematoxylin-eosin, 150 ×.

appeared in them. Meanwhile the superficial cortical layers of the lens fibers preserved their normal structure (Fig. 1).

The results show that lipid extracts of lenses from mice with cataract contained significantly more products with diene conjugation (lipids conjugated with hydroperoxides) and with characteristics fluorescence (LPO end products) than extracts from control lenses (Table 1). Typical spectra of excitation and emission of fluorescence of an extract obtained by Folch's method from lenses with cataract are illustrated in Fig. 2a. The maximum of excitation and emission lay at wavelengths of 365 and 430 nm, i.e., the spectral characteristics were those of nontryptophan fluorescence, the genesis of which is linked with fluorophores of protein nature [4].

To prove the lipid nature of the fluorescence discovered, the lipid extracts of lenses were fractionated on a column with Sephadex LH-20. It will be clear from Fig. 2b, which shows typical elution profiles of extracts of a lens with cataract, obtained by measuring the characteristic fluorescence and determining the phosphorus content in the fractions, that fluorescent products were eluted from the column in two peaks (high- and low-molecular-weight substances) with maxima corresponding to fractions Nos. 10 and 17 (curve 1). Phosphorus-containing fractions (curve 2) also were eluted in two maxima, but only one (fraction No. 10) corresponded to the maximum observed for fluorescence. The second maximum did not contain fluorescent products. On fractionation of a lipid extract from the brain of old rats, containing lipofuscin-like pigments, one maximum of fluorescent products, corresponding to the high-molecular-weight No. 10 (curve 3), was found in the elution profile. It can be concluded from a comparison of these data that the high-molecular-weight fluorescent fraction corresponds to peroxide products of phospholipid polymerization and that it is a lipid component of lipofuscin-like pigments [6]. What is the nature of the fluorochromes in the second fluorescent fraction, not containing phospholipids (phosphorus)? The answer to this question would seem to be all the more important because of fractionation of lipid extracts from the lenses of mice without cataract, only one peak of fluorescent products was recorded with a maximum in fraction No. 10, corresponding to products of lipofuscin-like nature (curve 4). The presence of the second peak of nonphospholipid fluorescent products in lipid extracts of mouse lenses is thus characteristic of cataract. To establish the precise nature of the product contained in the second fluorescent peak, high-resolution H^1-NMR spectra and mass spectra of this fraction were recorded. This showed that the highest molecular weight of the molecular ions obtained from substances contained in fraction No. 17 does not exceed 386, but signals from protons of various fatty acid groups and their oxidation products were recorded in the H^1-NMR spectra: the $-(CH_2)-$singlet at 1.63 ppm, methyl groups = CH_3 at 1.71-1.76 ppm, and methylene protons of a ketoaldehyde fragment at 3.48 ppm.

It can thus be tentatively suggested that fraction No. 17 contains "unpolymerized" oxidation products of free long-chain polyunsaturated fatty acids with molecular weight ($C_{22}H_{32}O_2$,

TABLE 1. Intensity of Fluorescence (I) and Degree of Oxidation of Lipids in Chloroform-Methanol Extracts of Mouse Lenses

Experimental conditions	I, conventional units per lens	D_{232}/D_{206}	D_{271}/D_{206}
Control	0,0102	0,182	0,031
Cataract	0,0147	0,219	0,052
Control	0,0022	0,208	0,017
Cataract:			
1	0,0096	0,250	0,073
2	0,0075	0,229	0,043

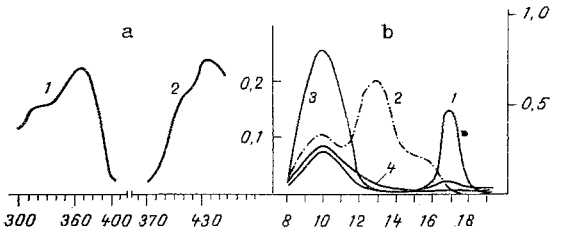

Fig. 2. Fluorescence excitation and emission spectra of lipid extracts from lens (a) and elution profiles of extracts (b). a: 1) Excitation spectrum (fluorescence recorded at 430 nm); 2) emission spectrum (wavelength of exciting light 360 nm). Abscissa, wavelength of light (in nm); ordinate, intensity of fluorescence (conventional units); b: 1) extract from lens with cataract; 2) phosphorus content in fractions after fractionation of lipid extracts from lens with cataracts; 3) brain extract from old rat; 4) extract of transparent lens. Abscissa, Nos. of fractions; ordinate, phosphorus content in sample (relative units).

i.e., $C_{22:6}$) of 328 + 2·32 (2 O_2). Addition of two molecules of oxygen gives a molecular weight close to that of the maximal molecular ion in fraction No. 17.

Signals from protons of the same aliphatic groups of lipids were found in the H^1-NMR spectra of the high-molecular-weight fraction, but in different ratios, and signals from protons of aromatic amino acids were not recorded. It can be concluded from these data as a whole that fluorescent products found in extracts from the lenses of mice with hereditary cataract are lipid in nature.

It must not be considered that the development of free-radical oxidation processes during cataract is confined to the lipid phase of the membrane structures of the lens. Active forms of oxygen and radical intermediates which can be generated interact also with protein components of the cells, as a result of which the accumulation of insoluble covalently cross-linked protein aggregates, characteristic of cataract, may take place and the content of monomeric forms of soluble and insoluble proteins is reduced [10].

Besides products of radical polymerization of phospholipids, products of fluorescent transformation of free polyunsaturated fatty acids, which have not undergone polymerization, also accumulate in the course of cataract development. The latter are probably formed with the participation of endogenous phospholipases of the A_2 type, capable of attacking both unoxidized and oxidized phospholipids [9].

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DOES α-TOCOPHEROL INTERACT WITH THE ACTIVE SITE OF CYTOCHROME P-450 IN LIVER MICROSOMES?

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Abundant experimental evidence of the universal role of lipid peroxidation in membrane damage following exposure to extremal factors and the development of various pathological states [4, 6] served as the basis for the use of inhibitors of free-radical oxidation, not only in the laboratory [10], but also in clinical practice [3].

As a result it has now become necessary to look for and screen synthetic antioxidants of low toxicity, with a prolonged stabilizing action in biomembranes [1]. This effect cannot be achieved through an increase in the concentration of antioxidants because of the toxic action which they exhibit [11] and of the oxidative metabolism of nonpolar synthetic inhibitors by the cytochrome P-450 system [5, 8]. Natural membranotropic antioxidants, such as tocopherols and ubiquinones, have a common structural principle: they have a cyclic nucleus with a hydroxy-group and a hydrophobic hydrocarbon "tail." The half-elimination time of natural antioxidants is much longer than that of their analogs without hydrocarbon substituents [9].

To test the hypothesis that hydrocarbon chains in the molecules of natural antioxidants play an essential role as a factor determining the length of their life in the membrane, a comparative investigation was undertaken of interaction of α-tocopherol (TP) and its synthetic derivative 6-hydroxy-2,2,5,7,8-pentamethylchromane (HPMC), which lacks the phytyl chain, with the cytochrome P-450 system, catalyzing the reaction of the first phase of biotransformation of the hydrophobic compounds.

EXPERIMENTAL METHOD

Intact noninbred albino rats weighing 150-180 g were used. The microsomal fraction of rat liver was isolated by differential centrifugation in 1.15% KCl. The concentration of cytochrome P-450 was determined by the method in [12]. Activity of microsomal 7-ethoxycoumarin de-ethylase was estimated from the velocity of the NADPH-dependent reaction of 7-hydroxycoumarin formation by the method in [15]. The spectra of binding of TP and HPMC with microsomes were recorded on a Hitachi-557 spectrophotometer by the method in [7]. TP and HPMC were added to a suspension of microsomes in ethyl alcohol or dimethyl sulfoxide so that the concentration

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