ISOLATION OF LIPOFUSCIN FROM BOVINE MYOCARDIUM

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Lipofuscin granules (LG) are structures found in the cells of most organs and tissues of animals and man which have been studied [7, 13, 15]. LP accumulation accompanies aging and is one of the characteristic features of many diseases [7, 13] and, in particular, of ischemia [5]. The structure and composition of LG have not been adequately studied and their functional role remains a matter for debate [7, 13-15]. Despite previous attempts [9, 15], no reliable methods are actually available for obtaining LG preparations.

In this paper we suggest a simple method for obtaining LG preparations from bovine myocardium. By means of this method preparative quantities of LG can be obtained (up to 10-20 mg of LG as protein from 100 g tissue), and the foundations are accordingly laid for research into the study of LG by physicochemical methods.

EXPERIMENTAL METHOD

The hearts of animals aged 5-7 years were used for isolation of LG. Operations for dissecting the heart and homogenization and isolation of LG preparations were carried out 1-2 h after sacrifice of the animals, at 2-4°C. The following media were used for isolation: solution A) 22% sucrose (w/v), 10 mM Tris-HCl (pH 7.6), 1 mM EDTA; solution B) 50% sucrose (w/v), 10 mM Tris-HCl (pH 7.6), 1 mM EDTA; solution C) 6% sucrose (w/v), 10 mM Tris-HCl (pH 7.6), and 1 mM EDTA. In the final stage of homogenization of the myocardium, a high-speed (14,000 rpm) tissue microblender (type 302, Poland) was used. The centrifugation procedures were carried out on centrifuges of the following types: J2-21 (Beckman, USA), in a JA-14 fixed-angle rotor (6 × 250 ml); VAC-601 (Janetski, East Germany) in a rotor with freely hanging cups (3 × 25 ml), and the K-24, in a fixed-angle rotor (12 × 11 ml) at 2-4°C. The protein concentration was determined by Bradford's method [10]. The spectral measurements were made on a Specord UV-VIS spectrophotometer (East Germany). Luminescence spectra were recorded by means of a microspectrofluorometer [3] at an excitation wavelength of 365 nm. Electron-microscopic analysis (EMA) of the preparations was carried out by the use of ultrathin sections and negative staining [6] on JEM-7A and JEM-100B microscopes (Heol, Japan).

EXPERIMENTAL RESULTS

The myocardium (200 g wet weight of tissue) was minced, mixed with 400 ml of solution A, and homogenized. The homogenate (600 ml) was mixed with 600 ml of solution B and centrifuged

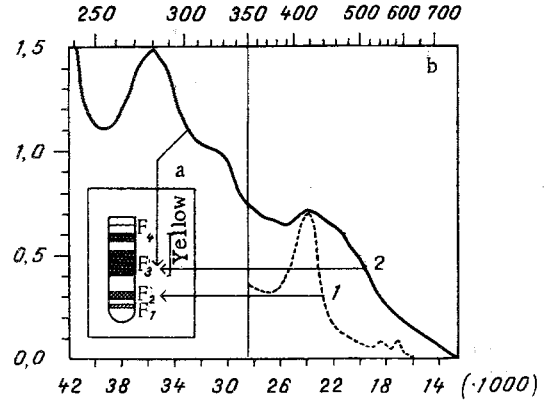

Fig. 1. Distribution of zones F_1-F_4 obtained by centrifugation of floating material in linear (15-45%, w/v) sucrose density gradient (a) and absorption spectra (b) of fractions F_2 (1) and F_3 (2). Abscissa: above — wavelength (in nm); below — the same, in cm^{-1}); ordinate, optical density.

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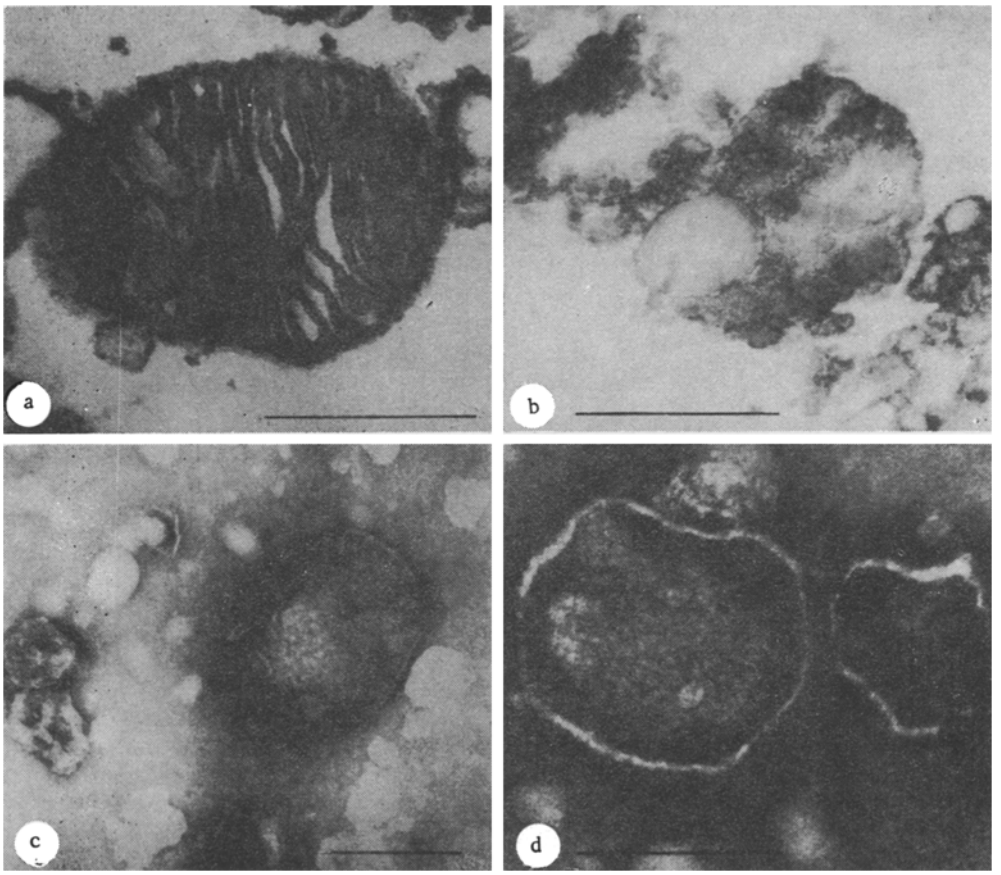

Fig. 2. Granules from fractions of F_2 (a) and F_3 (b-d) obtained by the use of ultrathin sections (a, b) and of negative staining (c, d). Scale, 0.5 μ.

(25,000g, 1 h) on a J2-21 centrifuge. The dark brown floating material was harvested, suspended in 4-8 ml of solution C, and sedimented (LK-24 centrifuge, 10,000g, 20 min). The residue was washed twice or three times with 10 ml of solution C at 10,000g for 20 min. In the final stage of isolation of the preparations the method of centrifugation in a linear sucrose of isolation of the preparations the method of centrifugation in a linear sucrose density gradient (15-45%, w/v) was used; the gradient was made up in medium containing 10 mM Tris-HCl (pH 7.6) and 1 mM EDTA. As a result of centrifugation (100,000g, 1 h) four opalescent zones (F_1-F_4) (Fig. 1a) were formed in the tubes, and two of them (F_3 and F_4) were yellow in color. The fractions were withdrawn by means of a syringe, suspended in 5 volumes of solution A, and centrifuged (10,000g, 30 min). The residues were suspended in 3-5 ml of buffer C and kept for not more than 3-7 days at 0°C (if longer keeping was necessary, the samples were cooled to between –20 and –40°C).

The protein concentration in fractions F_1 and F_2 was usually 5-10 mg/100 g wet weight of myocardium. The absorption spectra of these preparations in the visible region (Fig. 1b, 1) had an intensive narrow absorption peak in the 415-418 nm region and maxima at 540 and 580 nm, characteristic of heme-containing proteins [1], and they are evidently evidence that the main component of fractions F_1 and F_2 may be mitochondria and their fragments. Meanwhile, it was shown by EMA that these organelles constitute the main part of fractions F_1 and F_2 (Fig. 2a).

The total protein content of fractions F_3 and F_4 was 10-20 mg/100 g wet weight of myocardium. Usually protein content in fraction F_4 was less than in fraction F_3.

The absorption spectra (Figs. 1 and 2) of preparations F_3 and F_4 were similar. In the region of wavelengths from 350 to 430 nm a poorly defined absorption peak was observed with a maximum at 415-418 nm. The size of this peak varied from isolation to isolation, and it is evidence that fractions F_3 and F_4 are contaminated with traces of mitochondria or their enzymes. However, the possibility cannot be roled out that some of the heme-containing compounds in these fractions may also be of endogenous origin.

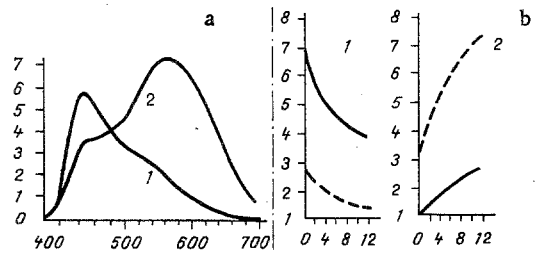

Fig. 3. Luminescence characteristics of LG preparations. Abscissa: a) wavelength (in nm); b) time (in min); a) luminescence spectra of floating material (1) and of fraction F_3 (2), change in intensity of luminescence of floating material (1) and of fraction F_3 (2) at wavelengths of 440 nm (continuous curve) and 550 nm (broken curve) under the influence of light with wavelength of 365 nm. Ordinate, intensity of luminescence (in relative units).

In the region of wavelengths of 430 and 600 nm severaly poorly resolved absorption peaks were reliably observed at 430, 460, and 490-500 nm. These spectral characteristics are typical of LG preparations from the myocardium of animals of this age, and in their shape in the visible region they closely resemble absorption spectra of single GL in the brain of old animals, obtained by other workers, according to whose data substances of carotenoid nature are among the components of LG [2, 14]. The results of the present experiments are thus evidence in support of the view that fractions F_3 and F_4 were in fact enriched with lipofuscin.

Absorption spectra of fractions F_3 and F_4 in the UV region were characterized by a maximum at 280-283 nm and by shoulders at 275 and 290-293 nm, and a minimum at 250-260 nm (Fig. 1b, 2). This is evidence that these LG preparations contain proteins with aromatic amino acids (mainly tryptophan and tyrosine) in their composition [1]. The optical density of the LG suspensions at 280 nm was 0.5-0.7, with a protein concentration of 1 mg/ml and an optical path of 1 cm.

The optical density of the LG preparations in the protein absorption band (280 nm) snd in the visible region was very close in value (Fig. 1b, 2). It it is assumed that absorption in the visible region is due mainly to carotenoids, with a molar extinction coefficient (E_M) of $8 \cdot 10^4$-$2 \cdot 10^5$ [12], and that E_M for protein is about 10^4-10^5 on average, it can be calculated that the molar concentrations of the carotenoids and protein in LG may be comparable. A rough calculation of this kind assumes that a significant part of the protein in LG can be in the form of protein-carotenoid complexes [8]. The possibility cannot be ruled out that the ratio of absorption in the ultraviolet and visible parts of the spectrum may characterize the degree of purification of the LG preparations. We know that a similar ratio is used to characterize the purity of preparations of the outer segments of the retinal rods, by far the greater part of whose proteins consists of rhodopsin — a complex of the membrane protein opsin with the carotenoid 11-cis-retinal [11].

According to EMA data (Fig. 2, b-d) fractions F_3 and F_4 consist mainly of granules measuing 0.5-3 μ. Their appearance and ultrastructure are similar to those of LG *in situ* [7, 9, 13, 15]. It was shown by negative staining method (Fig. 2c, d) that LH have an outer membrane about 10 nm thick. Evidently after injury to the granules, uranyl acetate penetrates inside and reveals the internal contents of the granules (Fig. 2d). A more detailed analyses of the results of electron microscopy of the F_3 and F_4 fractions may be the subject of a special examination.

It was shown previously [4, 14] that LG *in situ* processes visible luminescence with maxima at 440, 550-560, and 680 nm, during excitation by light with a wavelength of 365 nm. The ratios between the intensity of luminescence at these wavelengths are determined by many factors and, in particular, by age. The luminescence spectra of fractions F_3 and F_4 are closely similar and characterized by the presence of clearly defined maxima at 440 and 550-560 nm (Fig. 3a, 2). The similarity of the luminescence characteristics of the LG preparations and of LG *in situ* [5, 14] is evidently additional evidence in support of the view that the fractions F_3 and F_4 do in fact contain chiefly lipofuscin. In agreement with this view it was demonstrated that the level of luminescence of the LG preparations is increased by the action of exciting light (Fig. 3b, 2). A similar phenomenon is known to characterize the luminescence of LG *in situ* [4]. It was found in the course of the experiments that preparations of partially purified LG (floating material) unlike fractions F_3 and F_4, are characterized by short-wave (λ_{max} = 440 nm) luminescence, but the intensity of luminescence at 550-560 nm was relatively low (Fig. 3a, 1); the intensity of luminescence at 440 and 550 nm, moreover, fell under the influence of light with a wavelength of 365 nm (Fig. 3b, 1). This suggests that the luminescence method may be useful for verifying the purity of LG preparations during their isolation.

LG obtained by the method described above thus closely resemble, in all characteristics it is possible to compare, LG *in situ* [2, 4, 5, 7, 9, 13-15]. This encourages the hope that the preparations obtained (fractions F_3 and F_4) are actually rich in lipofuscin. The method examined above yields LG in quantities sufficient for their subsequent analysis by physicochemical methods.

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EFFECT OF STEROID AROMATASE INHIBITORS OH HYPOTHALAMIC CATECHOLAMINE CONTENT IN NEONATALLY ANDREGENIZED RATS

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It was shown previously that the defeminizing effect of testostrone on the developing brain of female rats is realized through accumulation of noradrenalin in the hypothalamus [3, 10]. Selective inhibition of catecholamine synthesis by injection of α-methyl-p-tyrosine prevents the development of anovulatory sterility in neonatally adrogenized rats [11]. A similar effect also was obtained by the use of aromatase inhibitors [4], confirming the important role of conversion of androgens into estrogens in androgen-dependent sexual differentiation of the brain (SDB) [8].

The aim of this investigation was to study the ability of aromatase inhibitors to prevent the rise in NA concentration in the hypothalamus induced by injection of exogenous testosterone into newborn female rats.

EXPERIMENTAL METHOD

Experiments were carried out on 140 newborn Wistar rats. Testosterone propionate (TP) was injected subcutaneously in a dose of 50 μg on the 5th day after birth. The aromatase inhibitors 4-androstene-3,6,17-trione (AT), in a dose of 0.5 mg, and 1,4,6-androstatriene-3,17-dione (ATD), in a dose of 1 mg (Steraloids, USA) were injected together with TP on the 5th day

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