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EXPERIMENTAL ARTICLES

Sterol Composition of the Arthrospores and Mycelium of the Fungus *Mucor hiemalis*

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Abstract—Sterol composition of the arthrospores and mycelium of the fungus *Mucor hiemalis* 1156 was studied by the method of chromatography–mass spectrometry. Along with ergosterol, the major sterol of the culture studied, ten minor sterols were identified, which were either precursors or products of ergosterol degradation. The content of individual sterols differed substantially in arthrospores and mycelium, which represent different stages of ontogenetic development of the fungus. In arthrospores, the content of ergosterol was lower than in mycelium (55.9 and 78.0%, respectively). Among the precursors of ergosterol, methylated sterols predominated in arthrospores (24.1% versus 11.6% in mycelium). Eburicol and 4,4-dimethylfecosterol were the major methylated sterols of arthrospores (10.6 and 8.1%, respectively). In addition, two uncommon and extremely rare sterols, 1-dihydro-dehydroneoergosterol and dehydroneoergosterol, were identified (for the first time in *M. hiemalis*). These substances, containing a complex system of conjugated double bonds in their A and B rings, are the products of ergosterol degradation. The data on sterol composition are discussed in terms of their morphogenetic implication.

Key words: lipids, sterols, ergosterol, mycelial fungi, arthrospores, dimorphism, *Mucor.*

Arthrospores of many fungal species are asexual propagative structures representing a separate stage of the fungal life cycle. They are formed mostly under conditions favorable for fermentation, as well as under aerobic conditions at a large content of acetate in the medium [1]. The fungus *Mucor hiemalis* 1156 grown under aerobic conditions on medium containing no inhibitors, produced arthrospores, which were round cells arranged in beaded chains at the tips of hyphae; arthrospores gave rise to yeast-like budding cells. Lipids of arthrospores differed from those of mycelial and yeast-like cells [2].

Under aerobic conditions, yeast-like growth of many fungi, including *M. hiemalis*, can be induced by the addition of antibiotics and fungicides [3–6]. Morphological changes were accompanied by changes in the fatty acid and sterol compositions. In particular, demethylase inhibitors, which block cleavage of the methyl group at C-14 in the lanosterol molecule, reduced the level of ergosterol and stimulated accumulation of C-14-methylsterols. As a result, physical and enzymatic properties of the membranes were affected, which was manifested in disturbed actin and chitin syntheses, as well as in abnormal chitin distribution in the cell wall [7]. In addition, aerobic fermentation with ethanol production was enhanced in the presence of the compounds inducing yeast-like cell formation in *M. hiemalis* [3].

Since changes in cell morphology are accompanied by changes in lipid composition, we proposed that, in addition to differences in the composition of fatty acids and certain lipid classes, the arthrospores and mycelium of the *M. hiemalis* 1156 may also exhibit differences in sterol composition.

In this study, the compositions of sterols and other lipids were compared in arthrospores and hyphae of the fungus *M. hiemalis* 1156 grown under aerobic conditions.

MATERIALS AND METHODS

This study used a culture of *Mucor hiemalis* 1156 from the All-Russia Collection of Microorganisms (VKM), Russian Academy of Sciences. An aqueous suspension of sporangiospores from a 14-day culture grown on wheat scalpings served as inoculum.

The strain was grown at 27°C for three days with shaking at 130 rpm in 250-ml flasks with 50 ml of medium of the following composition (g/l): glucose, 60; urea, 1; K_2HPO_4, 1; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; $ZnSO_4 \cdot 7H_2O$, 0.005; yeast extract, 0.5.

The mycelium was separated from the culture liquid by filtration through a glass filter no. 160 and, after resuspending in distilled water, it was separated from the arthrospores using a glass rod. Mycelium was again gathered by filtration, and arthrospores were collected

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Table 1. Results of chromatography–mass spectrometry of sterols isolated from the biomass of the fungus *M. hiemalis* 1156

No.	Sterol	Molecular mass	Retention time, min	Mass-spectrometric parameters, *m*/*z*
1	19-nor-24-methyl-cholesta-3,5(10),6,8(9), 22-pentaene-3β-ol (1-dihydro-dehydroneoergosterol	378	28.33	378, 363, **253***, **199**, 157, 143, 129, **91**
2	19-nor-24-methyl-cholesta-1,3,5(10),6,8(9),22-hexaene-3β-ol (dehydroneoergosterol)	376	31.00	**376**, 361, **251, 236, 197, 155, 141**, 129, **105**
3	24-methyl-cholesta-5,7,22-triene-3β-ol (ergosterol)	396	39.56	396, **363**, 337, 271, **253, 211, 157, 143**
4	24-methyl-cholesta-7,22-diene-3β-ol (5-dihydroergosterol)	398	40.94	398, 367, **271**, 213, **147**
5	24-methyl-cholesta-5,22-diene-3β-ol (7-dihydroergosterol)	398	40.98	398, 383, 365, **271**, 213, **145**
6	24-methylene-cholest-8-ene-3β-ol (fecosterol)	398	41.63	398, 383, 365, 199, **147**
7	24-methylene-cholest-7-ene-3β-ol (episterol)	398	42.45	398, 383, **314, 271**, 255, 246, 231, **161, 147**
8	24-methylene-4α-methyl-cholest-8-ene-3β-ol	412	44.16	412, 397, 355, **285**, 269, 253, 215, **161, 147, 109, 69**
9	24-methylene-4,14-dimethyl-cholest-5-ene-3β-ol	426	45.65	426, **411, 393**, 299, 282, 255, 243, **227**, 217, 213, 207, **171, 145, 107, 69**
10	24-methylene-lanost-8-ene-3β-ol (eburicol)	440	49.18	440, **425, 407**, 397, 299, **281, 259, 241**, 229, 215, **161**, 147, **109, 69**
11	14-nor-24-methylene-lanost-8-ene-3β-ol	426	50.22	426, 411, 408, 393, **299, 259**, 217, 203, **187, 173**, 159, **147, 135, 109, 69**

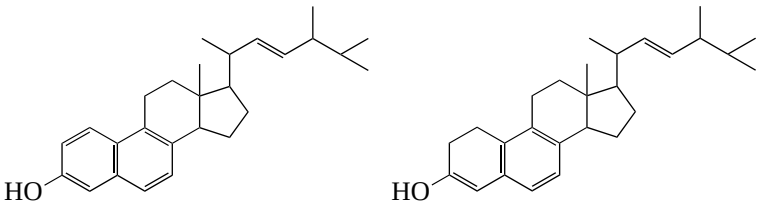
* The most intense fragments are boldtyped.

by centrifugation after microscopic examination for purity.

Lipid extraction was performed by the Folch method [8]. After alkaline hydrolysis of lipids at 80°C for 3 h in 5 ml of ethanol with the addition of 0.2 ml of 33% KOH, the unsaponifiable fraction was extracted with three portions of hexane, washed with distilled water, and dried with anhydrous sodium sulfate. The material obtained was dissolved in benzene and analyzed by chromatography–mass spectrometry followed by gas–liquid chromatography (GLC). Free fatty acids were extracted with hexane after acidification of an ethanol solution of saponified lipids with 6 N HCl. After evaporation, acidic methanolysis was conducted at 80°C for 1.5 h.

Fatty acid methyl esters were extracted with hexane to be analyzed by the GLC method on a model 3700 gas–liquid chromatograph (Russia). A column with 17% diethylene glycol succinate on Chromosorb W was used in an isothermal regime (175°C) at a carrier gas (helium) flow rate of 40 ml/min.

Sterols were analyzed on a Finnigan-3200 chromatograph–mass spectrometer (United States); the energy of ionizing electrons was 70 eV; the mass range was 40–500; the ion source temperature was 120°C. Chromatographic separation was carried out on a capillary column (0.25 mm × 50 m) of fused quartz with the SE-30 stationary phase; the temperature was 60°C for 2 min, 240°C for 8 min, and was further increased at a rate of 4 degree/min to 290°C. The evaporator temperature and the temperature of the transition lines was 280 and 250°C, respectively; the carrier gas (helium) flow rate was 1.2 ml/min. The retention time and the ratios of the characteristic ion peaks in the mass-spectrum were determined at the maxima of the peaks on chro-



(a) (b)

Fig. 1. Structure of uncommon sterols identified among lipids of *M. hiemalis* 1156: (a) dehydroneoergosterol; (b) 1-dihydro-dehydroneoergosterol.

Table 2. Relative content of sterols (% of the total) in arthrospores and mycelium of the fungus *M. hiemalis* 1156

No.	Sterol	Arthrospores	Mycelium
1	19-nor-24-methyl-cholesta-3,5(10), 6,8(9), 22-pentaene-3β-ol (1-dihydro-dehydroneoergosterol)	1.09	0.36
2	19-nor-24-methyl-cholesta-1,3,5 (10), 6,8(9), 22-hexaene-3β-ol (dehydroneoergosterol)	0.90	0.73
3	24-methyl-cholesta-5,7,22-triene-3β-ol (ergosterol)	55.87	77.96
4	24-methyl-cholesta-7,22-diene-3β-ol (5-dihydroergosterol)	0.25	traces
5	24-methyl-cholesta-5,22-diene-3β-ol (7-dihydroergosterol)	6.30	1.70
6	24-methylene-cholest-8-ene-3β-ol (fecosterol)	0.66	0.55
7	24-methylene-cholest-7-ene-3β-ol (episterol)	10.82	7.12
8	24-methylene-4α-cholest-8-ene-3β-ol (4-methylfecosterol)	2.19	0.94
9	24-methylene-4,14-dimethyl-cholest-5-ene-3β-ol	3.21	2.11
10	24-methylene-lanost-8-ene-3β-ol (eburicol)	10.61	6.85
11	14-nor-24-methylene-lanost-8-ene-3β-ol (4,4-dimethylfecosterol)	8.10	1.68
	Methylated/demethylated	0.32	0.13

matograms reconstructed by a computer. Sterols were identified by comparison of the mass spectra obtained with the published data, as well as using empirical correlations between the structural and mass-spectral characteristics of the compounds studied [9, 10]. Data on the consecutive chromatographic emergence of sterols with various substituents, as well as of their epimers and isomers with different positioning of conjugated bonds, were also taken into account. The relative content of sterols was calculated by standardization of the peak areas on a chromatogram of complete ionic flow, without taking the sensitivity coefficient into account.

The GLC analysis of sterol composition in the unsaponifiable fraction of arthrospores and mycelium was performed on a model 3700 chromatograph (Russia) using a capillary column, 0.25 mm × 60 m, with the SE-54 stationary phase; the temperature of the column was 290°C; the evaporator and detector temperatures were 300°C; the carrier gas (helium) flow rate was 1.5 ml/min.

Lipid classes were analyzed by thin-layer chromatography (TLC) on Kieselgel 60 F_{254} plates (Merk, Germany). To separate neutral lipids, a hexane–diethyl ether–acetic acid (80 : 20 : 1) system was used. The two-dimensional chromatography of polar lipids was conducted using a chloroform–methanol–28% ammonium hydroxide (65 : 25 : 5) solvent system in the one direction and a chloroform–acetone–methanol–acetic acid–water (6 : 8 : 2 : 2 : 1) system in another direction. Plates were developed with either a solution of 10% phosphomolybdic acid in ethanol or with sulfuric acid. Lipids were identified by qualitative reactions with ninhydrin (lipids with free amino group), α-naphthol (glycolipids), the Dragendorff reagent (choline-containing lipids), the Vas'kovskii reagent (phospholipids), and with a 1 : 1 mixture of sulfuric and acetic acids (free and esterified sterols), as well as by comparison with the R_f values of reference standards. Individual classes of lipids were determined quantitatively by densitometry.

RESULTS AND DISCUSSION

Eleven sterols were identified in the unsaponifiable lipid fraction from *M. hiemalis* 1156 by the method of chromatography–mass spectrometry. The results are shown in Table 1. Sterols of the culture examined were represented by ergosterol (the major sterol) and by its precursors, including methylated ones. Most of the identified compounds frequently occur in mycelial fungi and yeasts. Note that two uncommon sterols, 1-dihydro-dehydroneoergosterol and dehydroneoergosterol, previously not detected in fungi, were also identified. These sterols contain a complex system of conjugated double bonds in the rings (Fig. 1), and they have been very rarely found in eukaryotes [11]. Dehydroneoergosterol was previously detected in faeces of rats whose food allowance contained ergosterol [12]. Dehydroneoergosterol is the product of ergosterol transformation, which is metabolized in the rat organism to dehydroneoergosterol and nonsteroid products. These processes are probably a result of the intestine microflora activity. Some microorganisms are known to be able to induce cleavage of the 19th carbon atom and to introduce double bonds Δ 1, 3, 5(10), 7(8), 8(9) in the nucleus, which results in phenol-steroid formation (*Pseudomonas, Nocardia restrictis*); other microorganisms induce isomerization of the Δ7 bond in Δ6 (*Arthrobacter simplex*). In fungi, these reactions are less common, although they have been described in several species of *Fusarium, Rhizopus, Curvularia, Penicillium* and *Aspergillus* [13]. The products of ergosterol transformation may be involved in the morphogenesis of the *M. hiemalis* culture studied.

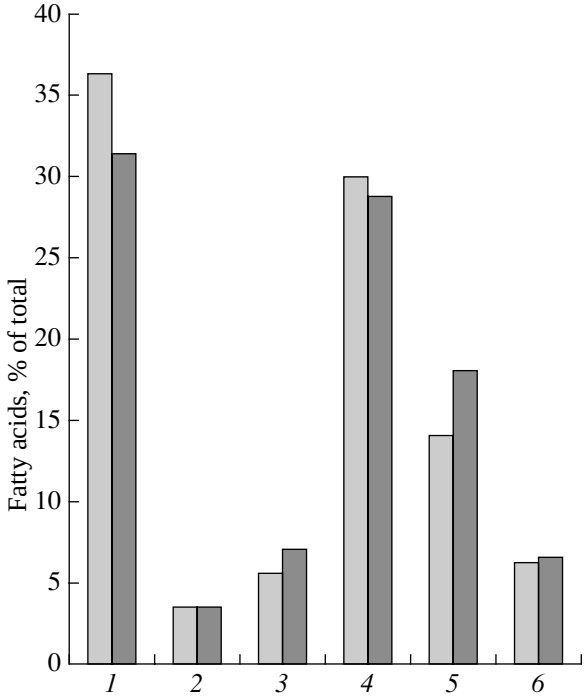


Fig. 2. Relative content of the fatty acids in lipids of mycelium and arthrospores of the fungus *M. hiemalis* 1156: (*1*) palmitic; (*2*) palmitoleic; (*3*) stearic; (*4*) oleic; (*5*) linoleic; (*6*) γ-linolenic acid. Light columns pertain to arthrospores; dark columns pertain to mycelium.

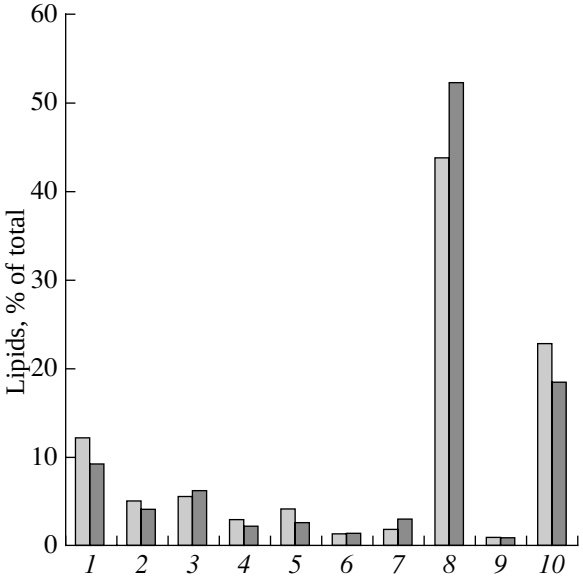


Fig. 3. Total lipid composition in arthrospores and mycelium of the fungus *M. hiemalis* 1156: (*1*) polar lipids; (*2*) diacylglycerols; (*3*) desmethylsterols; (*4*) methylated sterols; (*5*) free fatty acids; (*6*) unidentified fraction; (*7*) quinones; (*8*) triacylglycerols; (*9*) fatty acid esters; (*10*) steryl esters. Light columns pertain to arthrospores; dark columns pertain to mycelium.

The qualitative sterol composition of *M. hiemalis* 1156 was similar in mycelium and arthrospores, although substantial differences were recorded in relative content of individual sterols (Table 2). In arthrospores, the content of ergosterol was lower than in mycelium, and a higher level of minor desmethylsterols was observed (dehydroneoergosterol, 1-dihydro-dehydroneoergosterol, 5-dihydroergosterol, 7-dihydroergosterol, and episterol). In addition, the content of methylated sterols was increased in the arthrospores. These were 4α-methyl-fecosterol, 24-methylene-4,14-dimethyl-cholest-5-enol, and, especially, eburicol and 4,4-dimethyl-fecosterol; the latter methylated sterol is considered to be a degradation product of ergosterol precursors [6]. The proportion of 4,14-dimethylsterols in total sterols reached 13.82% in arthrospores and only 8.84% in mycelium.

Analysis of fatty acid composition in mycelium and arthrospores showed that they did not differ substantially in the relative content of individual fatty acids (Fig. 2). Nevertheless, in arthrospores, the level of saturated palmitic acid was higher and that of the linoleic acid lower than in mycelium, which resulted in a lower degree of lipid nonsaturation.

Arthrospores and mycelium also differed in their contents of individual classes of lipids (Fig. 3 and Fig. 4). In arthrospores, the content of triacylglycerols was decreased, and that of polar lipids, free fatty acids, and steryl esters was increased. The level of free sterols remained almost unchanged. In arthrospores, polar lipids contained more glycolipid-1 and phosphatidylserine and less phosphatidic acid, cerebroside, and phosphatidylethanolamine than in mycelium. The different lipid composition of arthrospores may be considered evidence of a lower intensity of metabolic processes.

Taylor and Parks [14] suggested that an increased level of steryl esters in total lipids is indicative of decelerated growth in response to depletion of nutrients in the growth medium. Many researchers assign esterified sterols to the reserve lipids. Steryl esters were shown to contain more lanosterol than free sterols, represented mostly by ergosterol [15, 16]. However, steryl esters can also protect the cell, in addition to serving as storage compounds [16].

A large portion of free fatty acids and ergosterol precursors, which are membrane-active compounds, can be bound via ester formation; after this, neither fatty acids nor ergosterol precursors can be further metabolized [16].

The reasons for arthrospore formation and the fine mechanisms underlying this process are still inadequately studied. Under conditions unfavorable for fungal growth, formation of sporangiospores is triggered. However, during submerged cultivation, formation of sporangiospores has never been observed in mucoraceous fungi. When propagation with sporangiospores is impossible, the ability to form arthrospores gains

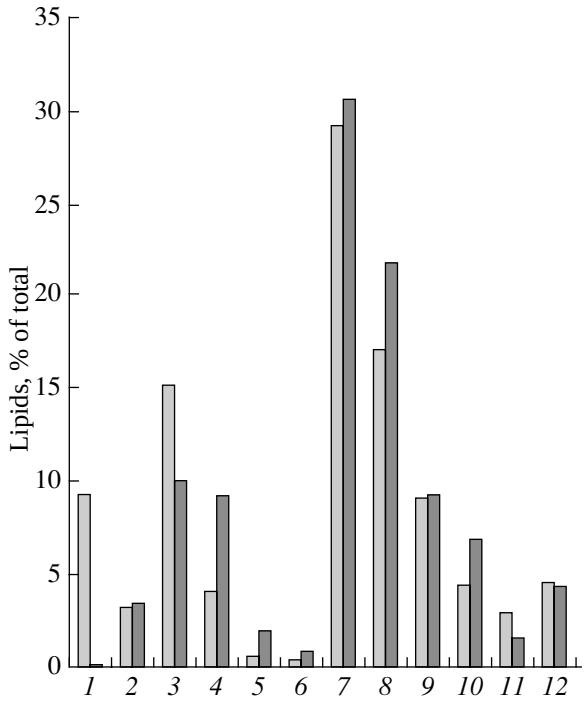


Fig. 4. Polar lipid composition in arthrospores and mycelium of the fungus *M. hiemalis* 1156: (*1*) glycolipid-1; (*2*) glycolipid-2; (*3*) phosphatidylserine; (*4*) phosphatidic acid; (*5*) glycolipid-3; (*6*) glycolipid-4; (*7*) phosphatidylcholine; (*8*) phosphatidylethanolamine; (*9*) cardiolipin; (*10*) cerebroside; (*11*) unidentified phospholipid; (*12*) glycolipid-5. Light columns pertain to arthrospores; dark columns pertain to mycelium.

importance. Arthrospore formation is an adaptive mechanism allowing a fungus to survive at a lower energy level. The change in the cell wall structure is considered the key event in the change of the morphology of the fungal cell. Hence, the role of membrane lipid composition is rather important, because it determines membrane fluidity and the activity of membrane-bound proteins. Treatment with low concentrations of fungicides inhibiting ergosterol biosynthesis led to abundant and abnormal chitin depositions in swallen regions of hyphae and septa; low content of ergosterol led to increased membrane rigidity [7]. In cells with plasma membranes deficient in ergosterol, the chitin-synthesizing activity was increased. These membranes most probably display higher fluidity, which may simulate turgor-induced membrane stress [17].

Ergosterol is the final product yielded by a complex chemical transformation of lanosterol, the major precursor of a large variety of sterols. The biosynthetic pathway includes: (1) a series of reactions for oxidative demethylation of the two methyl groups at C-4 and one at C-14; (2) methylation at C-24; (3) dehydrogenation, and (4) isomerization of the double bond Δ8 to Δ7 [18]. The methyl group can also be introduced into the side chain at C-24 prior to C-4 and C-14 demethylation, dehydrogenation, and isomerization, and this results in the formation of a triterpene eburicol [19]. The fact that sterols of *M. hiemalis* 1156 contain methyl and methylene groups at C-24 suggests that transmethylation precedes demethylation of the lanosterol molecule in the organism.

The double bond at C-24 is of great importance for ergosterol synthesis. At position 22, the double bond was detected only in 24-methylsterols (Table 1), which suggests that 24-methylene-sterol conformation prevents operation of the Δ22-desaturase of the side chain.

The increased level of 14-methylsterols in arthrospores of *M. hiemalis* 1156, especially the level of eburicol and 24-methylene-4,14-dimethylcholesterol, is indicative of a decreased activity of C-14-demethylase as compared to its activity in mycelium. Along with the increased content of palmitic acid and the decreased content of linoleic acid, this may lead to changes in the membrane viscosity [7]. Enhanced synthesis of saturated fatty acids is indicative of the effect produced on the desaturation enzymes that are closely related to the operation of the respiratory chain. In yeasts, the high proportion of methylated sterols and saturated fatty acids is known to be associated with a reduced level of heme-dependent enzymes [20]. The electron-transport chain consisting of cytochrome b_5 and cyanide-sensitive enzymes participates in the reactions of Δ5-desaturation of 5-dihydroergosterol, 4-demethylation of methylsterols, and Δ9-desaturation of palmitoyl-CoA; the electron-transport chain containing cytochrome *P* 450 is needed for Δ22-desaturation of the sterol side chain [20, 21]. The Δ22-desaturase of the side chain is inactive under anaerobic conditions, which is confirmed by the accumulation of 22-dihydroergosterol in *M. hiemalis* [22] and *S. cerevisiae* [21]. Upon adaptation to oxygen, the enzyme activity increases rapidly due to de novo synthesis of $P\ 450_{22DS}$ [21]. In arthrospores of *M. hiemalis* 1156, the content of unsaturated fatty acids and desmethylsterols decreased, whereas that of cardiolipin remained unchanged and no 22-dihydroergosterol was detected. We suggest, therefore, that in the arthrospores, the enzyme systems involving cytochrome b_5 are affected to a greater extent than those containing *P* 450.

Thus, analysis of the sterol composition of *M. hiemalis* 1156 showed that arthrospores differ from mycelium by a lower content of ergosterol, a higher content of intermediate products of ergosterol biosynthesis (including methylated ones), and by a higher content of the degradation products of ergosterol and its precursors.

According to the current concepts, sterols play an important role in the response of an organism to stressful conditions in the environment, which trigger morphogenesis in mycelial fungi. The differences revealed in the compositions of sterols, fatty acids, and individual classes of lipids may suggest a substantial role of lipids in the morphogenesis of *M. hiemalis* 1156.

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