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EXPERIMENTAL ARTICLES

# Lipid and Elemental Composition as Indicators of the Physiological State of Sporangiospores in *Mucor hiemalis* Cultures of Different Ages

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**Abstract**—The number of sporangiospores used as inocula may significantly affect the development of the morphogenetic programs in mucoraceous fungi, including manifestations of dimorphism. In order to assess the physiological state of sporangiospores differing in their viability and germination patterns, lipid composition of sporangiospores from 6-, 14-, and 20-day cultures of *Mucor hiemalis* F-1431 was determined and the element ratios in these spores (Ca/K and P/S) were measured using X-ray microanalysis. While the lipid composition of the "old" (20 days) and "young" (6 days) spores was generally similar, older spores contained no cerebrosides, had half the level of polar lipids and γ-linolenic acid, and also contained 6 times more monoacylglycerols and 2.5 times more phosphatidic acid. The ratio of esterified to free sterols (ES/FS) characterizing the sterol storage pool was lowest in the spores from 20-day cultures. X-ray microanalysis revealed the highest P/S ratio in 6-day spores and lowest ratio in 14-day ones. Mature 14-day spores had the highest Ca/K ratio, ten times exceeding that for the 20-day spores. Higher values of P/S and Ca/K ratios in young (ripening) spores than in old 20-day spores indicate their higher metabolic activity and correlate with their higher viability and mycelial type of germination. Together with the lipid characteristics, the Ca/K and P/S ratios are the parameters which may be used to develop the criteria for assessment of the physiological state of the cells, including viable fungal sporangiospores. This complex may be also used to assess the capacity of mucoraceous fungi for dimorphism, including the species (*M. hiemalis*) for which this capacity has not been demonstrated previously.

*Keywords*: *Mucor hiemalis*, sporangiospores, lipids, elemental composition, X-ray microanalysis, dimorphism

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Along with carbohydrates, lipids of the fungal spores are known to be a source of energy and building material used at the stage of germination. They are also involved in the regulation of biochemical processes [1], and their composition and quantity affect the choice of cell growth strategy [2]. According to the literature data, in some fungi the rate of hydration and age of spores influence their capacity for germination [3–5].

Our previous studies on *Mucor circinelloides* and *M. hiemalis* F-1156 demonstrated that at extended cultivation time a fraction of sporangiospores lost the capacity for germination/viability, while the spores retaining this capacity commenced yeastlike growth, apart from mycelial growth. Therefore, sporangiospores in the cultures of different ages possessed varying ability to exhibit dimorphism: sporangiospores of young cultures (5–6 days) initiated only mycelial growth upon germination, while the spores of old cultures (19–20–25 days) exhibited mainly yeastlike growth [6–8]. Based on the studies of spore lipids, changes in their composition were concluded to be a possible indicator of the physiological condition/viability and of the extent of their dimorphism.

Viability of microbial cells is known to depend on the state of intracellular components and metabolic systems and depends on many external factors. X-ray microanalysis is one of the methods suitable for analysis of ionic homeostasis in various biological objects [9, 10]. In particular, the method was used to reveal the differences in the content of individual biogenic elements (S, P, Ca, and K) and their ratios (Ca/K and P/S) in the cells of various microorganisms (*Bacillus cereus, Micrococcus luteus, Saccharomyces cerevisiae)* differing in their metabolic and proliferative activities according to the following sequence: vegetative cells–viable dormant forms–nonviable cells [11]. The elucidated differences in the elemental content and ratio in the dormant forms probably reflect the changes in

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their ionic homeostasis upon cell transition to an anabiotic state and may be used to develop the criteria for diagnosis of the physiological status of microorganisms. The methods used currently, such as determination of calcium in living fungal cells, are complex [12] and their application in routine analyses is labor-consuming; therefore, application of X-ray microanalysis for evaluation of the viability of fungal spores by the ratio of major elements seems feasible. This method makes it possible to determine the atom ratios even in individual cells (including the spores), which allows for statistically reliable values characterizing the state of the culture in general.

The goal of the present work was to study the changes in lipid and elemental composition, as well as the morphology of *Mucor hiemalis* F-1431 sporangiospores of various ages, in order to evaluate their physiological state and capacity for dimorphism.

## MATERIALS AND METHODS

**Organism.** The fungal strain used in the work, *Mucor hiemalis* F-1431, was obtained from the All-Russian Collection of Microorganism, Russian Academy of Sciences.

**Cultivation.** Solid-phase cultivation of the sporogenic culture of the fungus on wheat bran in 2-L flasks for 6, 14, and 20 days at 27°C was used to obtain sporangiospores. The bran (15 g) placed in each flask, supplemented with 20 mL distilled water, and sterilized at 1 atm. The bran was inoculated with the spore suspension (5 mL) obtained by washing off the surface of the sporogenic mycelium grown for 7 days in test tubes on wort–agar slants. When the culture reached the desired age, sporangiospores were washed off the surface of mycelium with sterile distilled water and collected by centrifugation. The obtained spore material was studied by epifluorescence and electron microscopy, as well as by X-ray microanalysis.

**To assess the viability of sporangiospores** of *M. hiemalis* F-1431 of different age by light microscopy, an Axio Imager.D1 (Carl Zeiss, Germany) phase contrast microscope was used at 400× magnification. In the work, the Plant Cell Viability Assay Kit (Sigma, United States) containing the fluorescent dyes fluorescein diacetate and propidium iodide was used according to the manufacturer's protocol.

**Electron microscopy** of ultrathin cell sections upon contrasting and staining according to Reynolds was performed using a JEM-100CXII (Jeol, Japan) electron microscope at 20000× magnification.

**X-ray microanalysis of the elemental composition** was performed using a JEM-100CXII (Jeol) electron microscope equipped with an EM-ASID4D scanning accessory and an X-ray microanalyzer (Green Star, Russia) at 60 keV. Suspensions of sporangiospores washed off the 6-, 14-, and 20-day cultures of *M. hiemalis* F-1431 and resuspended in deionized water were used for analyses. The spore suspension was applied onto copper grids (3 mm in diameter) with carbonized formvar film; the preparations were air-dried for 24 h and carbon-plated at 90°. The spectra were processed with the LAF/PB software package. The ratios of Ca/K and P/S were calculated from the areas of the relevant peaks.

**Extraction of lipids.** Lipids of the sporangiospore biomass were extracted according to Folch [13] and subjected to acidic methanolysis in a mixture of methanol and acetyl chloride at 80°C during 1.5 h. The ratio of the components in the methanolysis mixture was 50 : 4.2. Methylation completeness was controlled by thin layer chromatography in a hexane–diethyl ether–acetic acid, 80 : 20 : 1, mixture. To study lipid composition of sporangiospores of different age, methods of gas–liquid and thin layer chromatography were used.

**Fatty acid methyl esters were analyzed by gas–liquid chromatography (GLC)** on a Khromatek Kristall-5000.1 (Russia) chromatograph using an Optima-240, 60 m × 0.25 mm × 0.25 μm capillary column (Macheray-Nagel Gmbh & Co., Germany) in the 33% cyanopropylmethyl–67% dimethylpolysiloxane stationary phase, under programmed mode at the column temperature gradient from 130 to 280°C and carrier gas (helium) flow of 30 mL/min.

**Composition of the lipid classes was determined by thin-layer chromatography (TLC)** on Kieselgel 60 $F_{254}$ plates (Merck, Germany). The following solvent systems were used for TLC: hexane–diethyl ether–acetic acid, 80 : 20 : 2, for neutral lipids; chloroform–methanol–water, 65 : 25 : 4, for polar lipids in the first direction, and chloroform–acetone–methanol–acetic acid–water, 50 : 20 : 10 : 10 : 5, in the second direction. Lipids were detected using 10% phosphomolybdic acid in methanol upon heating. Lipid identification on TLC plates was performed by comparison of their $R_f$ values with those of the standards (Sigma, United States), as well as by qualitative reactions with ninhydrin (to identify the lipids with free amino group), α-naphthol (to identify glycolipids), Dragendorff's reagent (to identify choline-containing lipids) [14], Vaskovsky's reagent (to identify phospholipids) [15], and the mixture of sulfuric and acetic acids, 1 : 1 (to identify free and esterified sterols) [14]. Densitometry analysis was performed using the Dens and Sorbfil (Russia) software packages. Standard solutions of phosphatidylcholine, triacylglycerol, and free fatty acids were used to build the calibration curves.

**Statistical processing of the results** was performed using the median method, as well as with the Microsoft Office Excel 2007 package. Average results of four independent experiments are reported.

## RESULTS

**Studies on the viability of sporangiospores of various age.** To study the effect of the condition of the sporangiospores used as inoculate on realization of the mor-

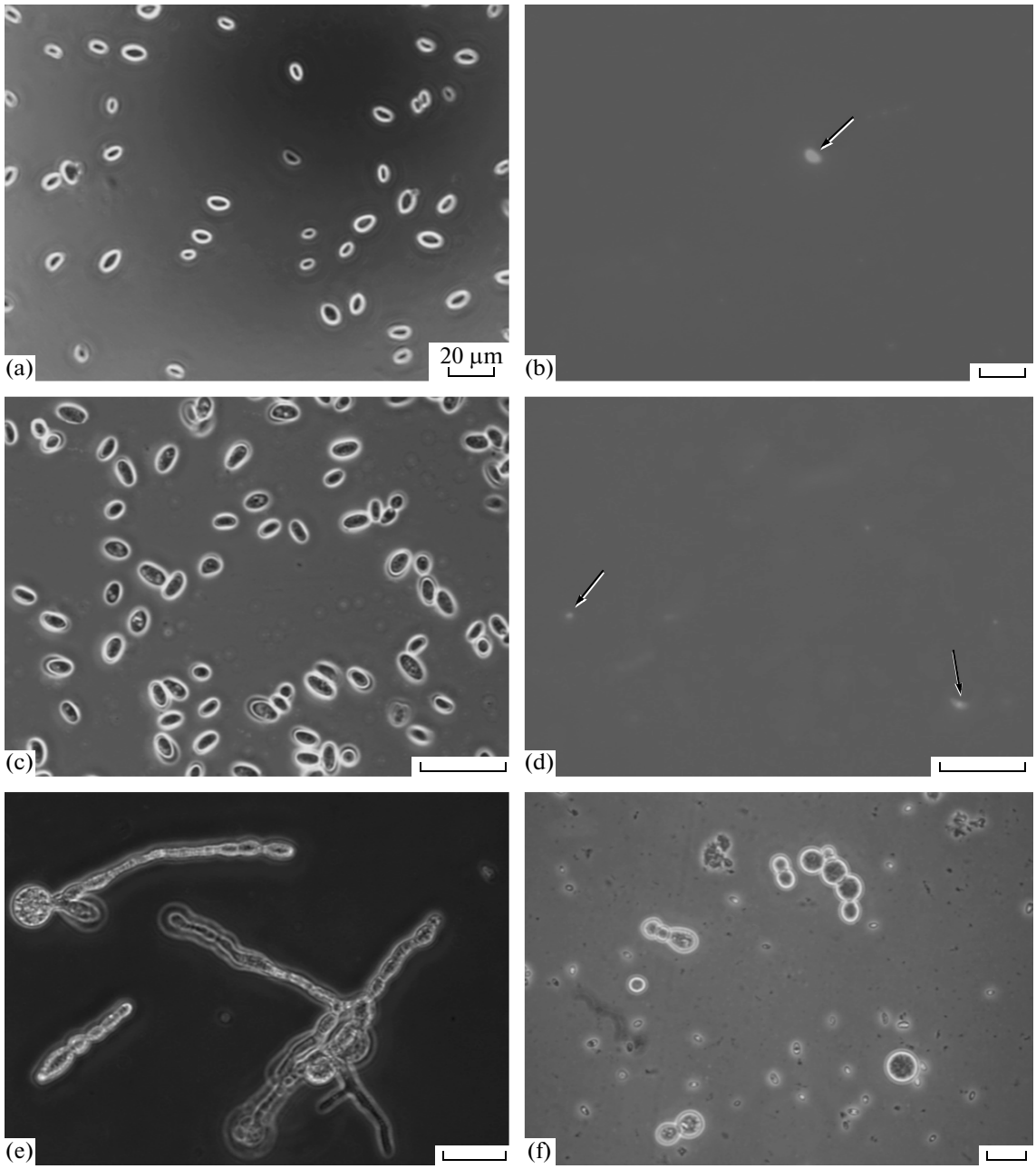


**Fig. 1.** Sporangiospores from the 6-day (a, b) and 20-day (c, d) cultures of *M. hiemalis* F-1431. Phase contrast (a, c); PI staining, nonviable cells are stained red (indicated with arrows) (b, d). Mycelial growth upon germination of the 6-day culture sporangiospores (e) and yeast-like growth of the 20-day culture spores (f); multiple ungerminated spores are observed; phase contrast. Scale bar, 20 μm.

phogenetic programs (dimorphism phenomenon) in mucoraceous fungi, the previously unstudied strain *Mucor hiemalis* F-1431 was used. The obtained spore material of 6- and 20-day cultures was analyzed using fluorescence dyes; however, treatment with fluorescein diacetate (FDA) did not result in unambiguous identification of viable cells, probably due to specific features of the sporangiospore cell wall structure. Inaccuracy of FDA as a spore viability indicator has been reported in the literature previously [16]. Application of propidium iodide (PI) made it possible to determine the percent of nonviable cells by their bright-red coloration (Fig. 1). No fatal loss in the viability of the spores from old cultures was observed. Among the sporangiospores of the 20-day sporogenic culture, dead cells constituted only 3.11% of the total number, that is, the amount of dead cells increased not more than two times, compared to the spores of the 6-day culture (3.11 and 1.85%, respectively). Apparently, the spores of the old culture were either in

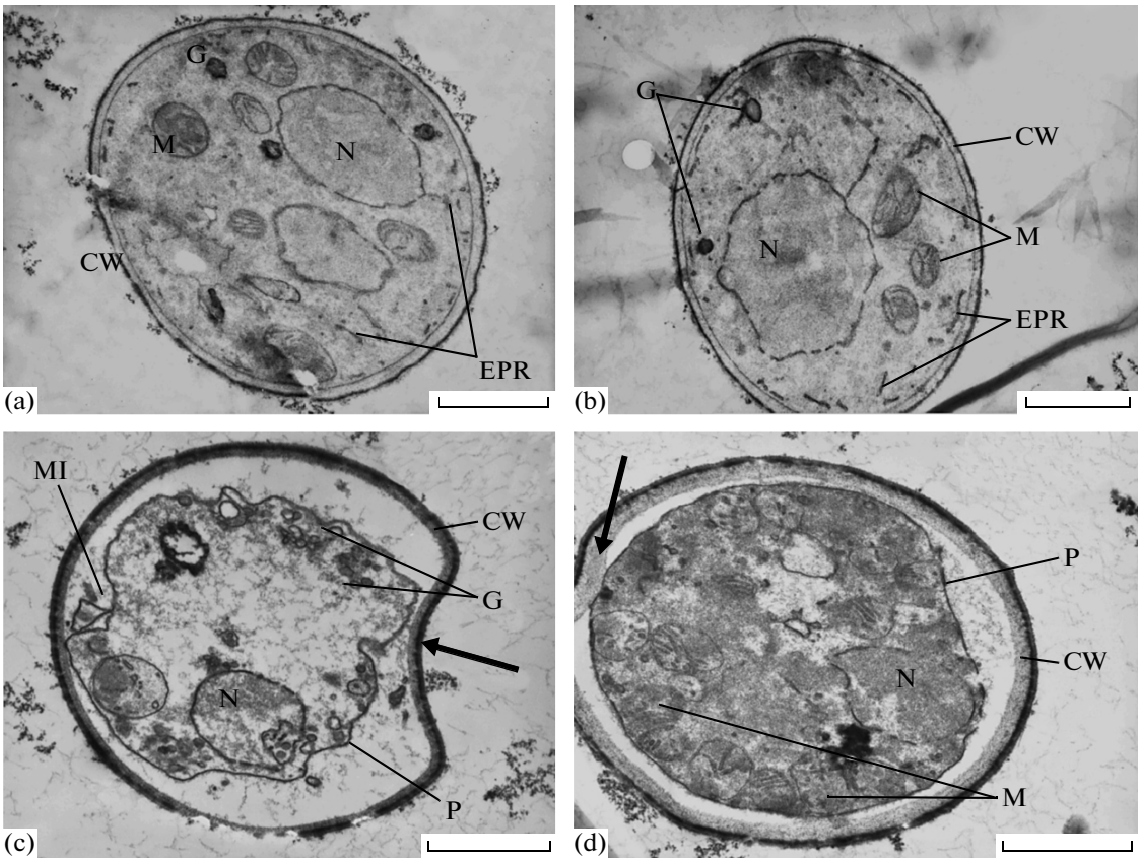


**Fig. 2.** Thin sections of sporangiospores from the 6-day (a, b) and 20-day (c, d) culturse of *M. hiemalis* F-1431. Scale bar, 2 μm. Designations: N, nucleus; M, mitochondria; CW, cell wall; EPR, endoplasmic reticulum; G, storage granules; MI, membrane invaginations; MV, microvesicles; P, protoplast; arrows indicate invaginations of the cell wall.

a deeper dormant state (without loss of viability) or possessed a dense and firm (undamaged) cell wall preventing the dye penetration inside the cell.

**Electron microscopic investigation of the *M. hiemalis* F-1431 sporangiospore ultrastructure** showed that the spores of the 6-day culture (Figs. 2a, 2b) had a smooth cell wall (CW) and were well hydrated—the protoplast and the cytoplasmic membrane adhered tightly to the CW internal surface. The nucleus, membranes of the cytoplasmic reticulum, and storage granules were well distinguished; mitochondria had multiple cristae.

Changes in the membrane apparatus were characteristic of the sporangiospores of the old, 20-day culture (Figs. 2c, 2d): numerous membrane invaginations were observed; intracellular membranes and cytoplasm content degraded, many small membrane vesicles appeared; the cells were dehydrated and plasmolysed, with the protoplast separated from the CW. Despite the individual invaginations of the cell walls in old spores (Fig. 2c, 2d), the cells retained their integrity. The mitochondria were also preserved, although with fewer cristae (Figs. 2c, 2d). Electron microscopy results supported the conclusion made from the fluorescence microscopy data that cell wall in the old spores of *M. hiemalis* F-1431 remained intact (or undamaged).

**Changes in the elemental composition of sporangiospores of different ages.** Investigation of sporangiospores of *M. hiemalis* F-1431 6-, 14-, and 20-day cultures by X-ray microanalysis was performed in order to evaluate the physiological state of sporangiospores with varying capacity for dimorphism. X-ray microanalysis provides significant information on the correlation between the metabolic activity and elemental composition of the cells [11]. The following elements were of most interest: calcium, as a secondary cell effector and stabilizer of biological macromolecules and membranes; potassium, as an element involved in creation of the transmembrane potential, water exchange, and maintenance of osmotic pressure in the cell; sulfur, as a protein component; and phosphorus, as an element comprising the nucleotides (ATP and others), nucleic acids, phospholipids, and energy equivalents. The ratios of Ca/K and P/S, according to our working hypothesis, should reflect the specific features of the elemental composition of young and old spores, which are probably different in their potential proliferative activity/viability and capacity for dimorphism.

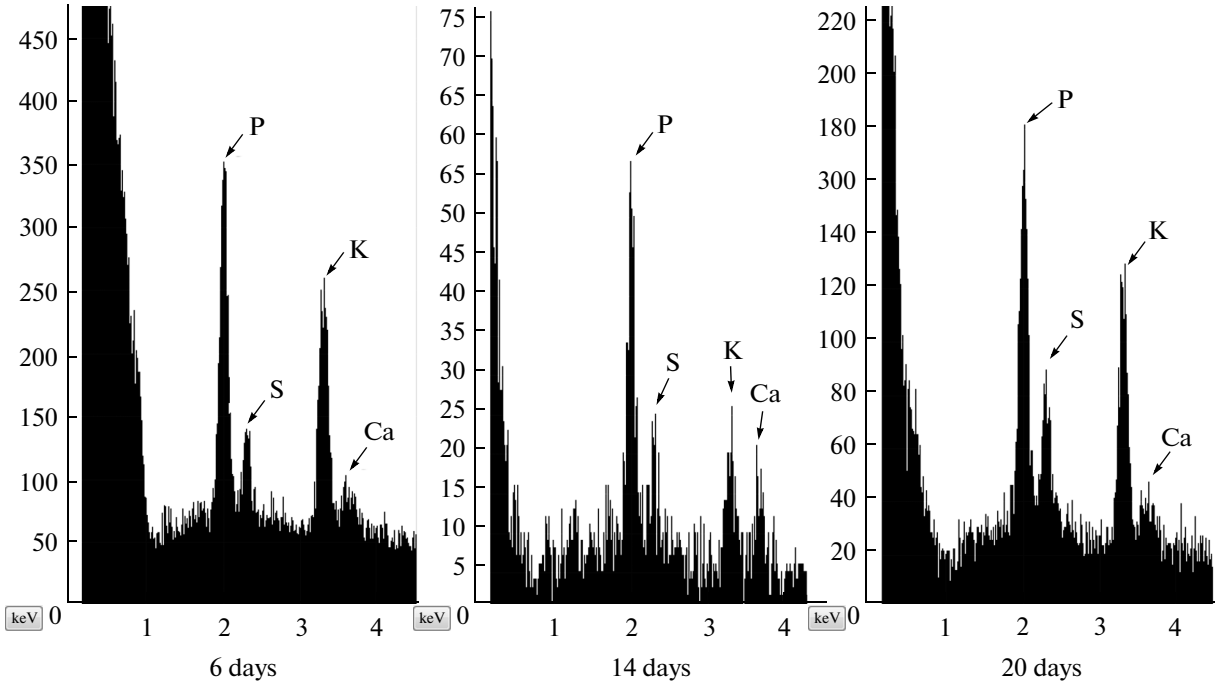


**Fig. 3.** Spectra of elemental composition of sporangiospores of *M. hiemalis* F-1431 cultures of different ages: 6 (a), 14 (b), and 20 days (c).

It was shown that the spectra of elemental composition of the spores of different age were rather similar (Fig. 3). Nevertheless, upon quantitative treatment of the data, the ratio of phosphorus and sulfur (P/S) was found to be the highest in the spores from 6-day cultures and the lowest in those from 14-day cultures (Table 1). We assume that this may evidence the changes in the content of phosphorus-containing compounds, including phospholipids, in the total lipid pool, and predomination of the storage lipids in the biomass. After 14 days, the catabolic processes accompanied by a decrease in the total lipid fraction, degradation of the membranes, and exhaustion of the storage compounds (for example, polyphosphates), with the overall preservation of the proteinaceous constituents of cells, probably start to prevail in sporangiospores.

Mature 14-day spores had the highest value of Ca/K among the studied variants. An almost tenfold decrease of this ratio in 20-day spores compared to the 14-day spores may be explained by the dehydration of the cells and changes in their water exchange. This may lead to destabilization of the membrane structures and, consequently, loss of the ability to germinate.

**Table 1.** Ratio of elements in sporangiospores of *M. hiemalis* F-1431 from the cultures of different age

| Ratio of the elements | 6 days | 14 days | 20 days |
|---|---|---|---|
| P/S | 8.080 | 2.244 | 5.071 |
| Ca/K | 0.117 | 0.626 | 0.067 |

Thus, the high values of P/S and Ca/K in the young (ripening) 6-day spores, if compared to the values in the old 20-day spores, characterize the former ones as potentially more metabolically active structures and correlate with their high viability and exclusively mycelial type of growth upon germination (Fig. 1e).The 14-day age of the sporogenic culture is probably the turning point in the process of formation and ripening of *M. hiemalis* F-1431 sporangiospores (which is reflected by their elemental composition). After reaching this turning point, the ratios between the major elements in spores change. When old sporangiospores were used as inocula, their capacity for germination, particularly via the mycelial type, decreased significantly, and formation of yeastlike cells began (Fig. 1f). Therefore, we suppose that the values of P/S and Ca/K in sporangiospores of different ages reflect the level of proliferative potential of the cells and the state of the intracellular structures and may be used for characterization of the spore ability to germinate and to exhibit dimorphism.

**Comparative study of lipid composition in sporangiospores of different age.** Sporangiospores of *M. hiemalis* F-1431 washed off the surface of 6-, 14-, and 20-day sporogenic cultures had different lipid contents (Table 2). The highest level of total lipids in dry biomass was characteristic of 14-day spores, while the

spores of young and old cultures were characterized by low content of total lipids.

We assume that the change (decrease) in the ability to germinate and the distinct capacity for yeast-like growth may be caused by the biochemical processes proceeding in sporangiospores of the old culture, including the exchange of polar (membrane lipids), as well as neutral (storage) lipids represented by triacylglycerols and sterol esters (TAGs and ESs, respectively).

Among the fatty acids (FA) of total lipids, compounds with 16 and 18 carbon atoms in a chain with varying content of double bonds prevailed (Fig. 4). Minor amounts of long-chain FAs (20–24 carbon atoms in chain) were also noted (4.76–2.25%). The level of lipid unsaturation in the spores (Table 2) was in inverse relation with their content: sporangiospores of 14-day cultures, having the highest content of lipids, were characterized by the lowest unsaturation levels, that is, increased level of saturated FAs (palmitic and stearic) and the lowest level of γ-linolenic acid (Fig. 4). It should be noted that no considerable changes were observed in the content of mono- and diene acids independently of the sporangiospore age. The highest values of unsaturation were typical of the lipids of young (6 days) spores (Table 2).

Neutral lipids of the sporangiospores of the strain under study were represented by mono-, di-, and triacylglycerols (MAG, DAG, and TAGs, respectively), free sterols (FS), free fatty acids (FFA), esterified sterols (ES), and unidentified fractions (X1 and X2) (Fig. 5). With the age of sporogenic cultures, the relative content of polar lipids (PL) and TAGs decreased in sporangiospores, which was accompanied by an increase in the MAGs fraction. In 14-day spores the highest ES content (22.4%) was noted; it decreased in 20-day spores to 14.6%. The ratio of esterified and free sterols (ES/FS) was also the highest in the lipids of 14-day spores (1.54, 2.33, and 1.15 for spores of 6-, 14-, and 20-day cultures, respectively). The level of free sterols changed insignificantly.

**Table 2.** Content and degree of unsaturation of the lipids in sporangiospores of *M. hiemalis* F-1431 cultures of different age

| Parameter | 6 days | 14 days | 20 days |
|---|---|---|---|
| Lipids, % to dry biomass | 3.89 | 12.53 | 3.0 |
| Degree of unsaturaiton, Δ/100 FA molecules | 150.96 | 67.38 | 129.0 |

Polar lipids contained phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), phosphatidylglycerol (PG), diphosphatidylglycerol (DPG, or cardiolipin, CL), glycolipids (GL, including two fractions of cerebrosides), and a number of unidentified minor fractions (X1 and X2) (Fig. 6). The age-related changes in polar lipid composition in the spores included a decrease in cerebroside content (from 13.3% totally to 0), some decrease in PE fraction, and a considerable increase in the level of PAs (from 7.36 to 18.55%). The level of one of the major bulk phospholipids, PC, in the spores of the 14-day culture was the lowest (15.57%) if compared to the spores of younger and older cultures (24.6 and 23.69%, respectively). The ratio of bulk phospholipids (PE/PC) in the spores was 0.77, 1.21, and 0.64, respectively. Content of CL, the major phospholipid of the mitochondria membranes, remained practically unchanged, which is in agreement with the electron microscopy data on the state of mitochondria in the cells.

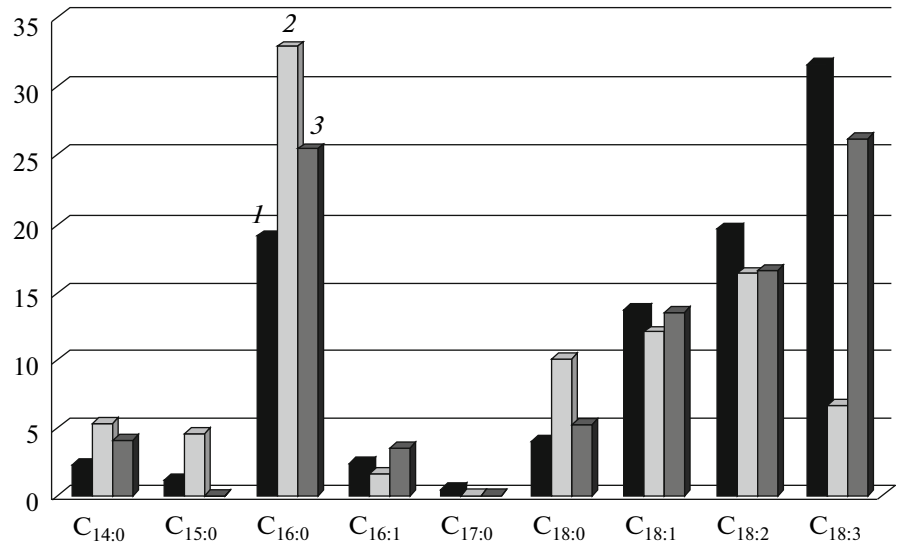


**Fig. 4.** Composition of major fatty acids of the total lipids in sporangiospores from *M. hiemalis* F-1431 cultures of different ages (% to total fatty acids): 6 days (*1*), 14 days (*2*), and 20 days (*3*).

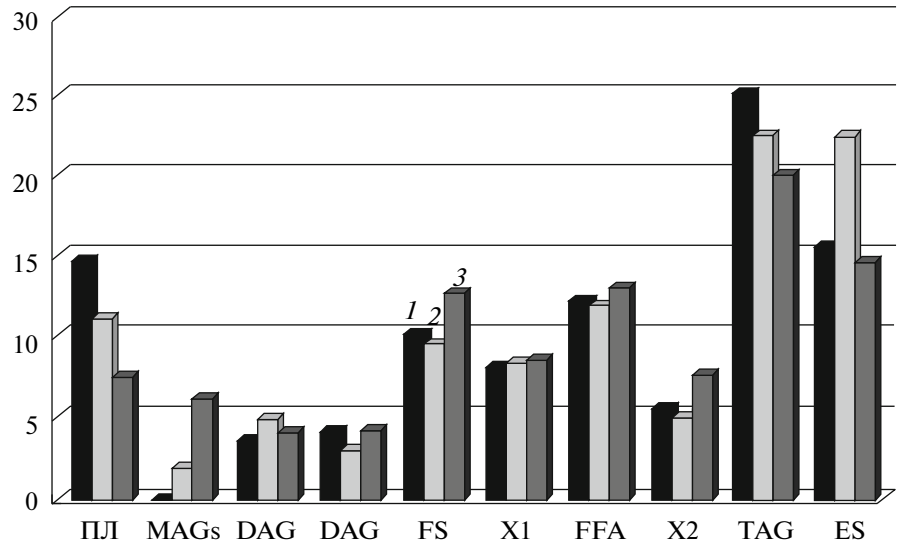


**Fig. 5.** Composition of lipid classes in sporangiospores from *M. hiemalis* F-1431 cultures of different ages (% to total): 6 days (*1*), 14 days (*2*), and 20 days (*3*). PL, phospholipids; MAGs, monoacylglycerols; DAG, diacylglycerols; TAG, triacylglycerols; FS, free sterols; FFA, free fatty acids; ES, sterol esters; X1, X2, unidentified fractions.

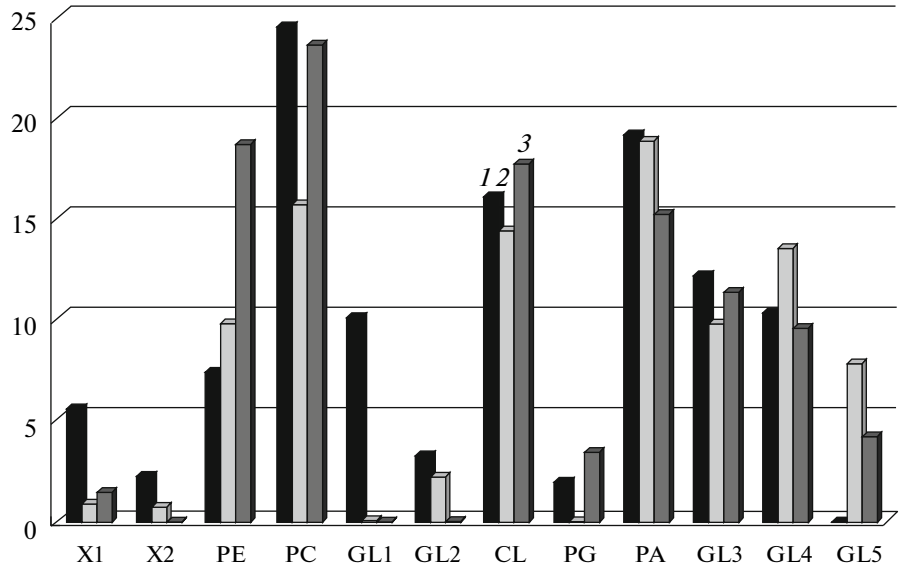


**Fig. 6.** Composition of polar lipids in sporangiospores of *M. hiemalis* F-1431 cultures of different ages (% to total): 6 days (*1*), 14 days (*2*), and 20 days (*3*). PE, phosphatidyl ethanolamine; PC, phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; CL, cardiolipin; GL, glycolipids (including two fractions of cerebrosides); and X1, X2, unidentified fractions.

## DISCUSSION

Investigation of the lipid composition of sporangiospores in *Mucor hiemalis* F-1431 cultures of various age evidences considerable changes in the course of spore ripening and aging. Spores of young 6-day cultures (arbitrarily termed "young spores") have low content of cell lipids characterized by high degree of unsaturation due to high content of γ-linolenic acid, increased level of polar lipids with low content of PA, and high content of bulk phospholipids (PC and PE), as well as relatively high content of storage lipids (TAGs and ES). Upon germination, such spores form growing tubes and give rise to mycelial growth exclusively (Fig. 1e). Mature spores of the 14-day culture are characterized by the most pronounced accumulation of lipids with high content of storage TAGs and especially ESs. These lipids have low degree of unsaturation caused by low content of γ-linolenic acid and high level of palmitic and stearic acids. Polar lipids of mature spores contain less bulk phospholipids than young spores. The lipid composition of mature spores (14-day culture) may reflect the turning point, after

which catabolic processes begin to dominate in the cells. Spores of 20-day cultures (arbitrarily termed "old spores") were characterized by exhaustion of the lipid pool, in which the content of neutral storage lipids (TAG and ES) decreased, as well as that of the polar membrane lipids. In general, the lipid composition of old and young spores possessed certain similarity, although in the lipids of old spores the content of polar lipids and γ-linolenic acid was two times lower, content of MAGs increased sixfold and that of phosphatidic acid increased 2.5 times, while cerebrosides were absent. The ratio of ES/FS characterizing the storage pool of sterols was the lowest (1.15) in old spores if compared to the same values for the young 6-day spores and mature 14-day spores (1.54 and 2.33, respectively).

Sporangiospores of mucoraceous fungi are not expected to survive prolonged exposure to extreme conditions, being the propagative structures necessary for rapid colonization of new substrates. After ripening of the spores (14 days, when the spores contain the maximum amount of lipids), new lipid are not synthesized, ionic homeostasis is impaired, destructive processes begin to dominate, and, probably, metabolites capable of the regulatory effects on further growth and germination begin to form. In the strain under study (*M. hiemalis* F-1431), in old (over 14 days) spores the ability to germinate (viability) and to form mycelium decreased sharply, while the capability of yeastlike growth appeared and was especially pronounced in the sporangiospores from the 20-day culture. Therefore, the data obtained agree with the results of previous studies on lipid composition of the spores of various mucoraceous fungal species [6–8], on the basis of which one may conclude that fulfillment of the morphogenetic programs (including dimorphism) depends on the changes in lipid composition of sporangiospores and their physiological condition.

Together with the structural and morphological characteristics and specific features of elemental composition described above, emergence of considerable amounts of PA among the lipids of old spores can affect viability and morphogenesis of the spores. Phosphatidic acid is known to be one of the secondary messengers—signal compounds of lipid nature involved in the regulation of morphogenetic processes and various cell functions [17–19]. In addition to its involvement in the processes of signal transduction, PA, being a component of the cell membranes among anionic phospholipids, is characterized by the ability to induce formation of microdomains, leading to development of membrane invaginations of negative curvature, which promote formation of microvesicles [20]. Increase of the PA fraction in old spores of *M. hiemalis* F-1431 correlates with the appearance of numerous membrane invaginations and vesicles revealed by electron microscopic investigation, which evidences the fulfillment of both sparking and bulk functions of PA.

Fungal asexual spores function as the structures providing for rapid dissemination of a species, rather than for prolonged survival, and are therefore in a state of shallow dormancy. When removed from mycelium surface, they may be stored without loss of quality (germination capacity) for a long time [3, 21]. According to the results of our studies, in the spores left on the mycelium, metabolic processes affecting lipid exchange occur, which lead to a considerable worsening of the sporangiospore quality. Therefore, research on the model of sporangiospores of *M. hiemalis* F-1431 cultures with various levels of capacity for dimorphism showed that their lipid composition determined sporangiospore quality to a considerable extent and played an important role in realization of various cell growth strategies.

On the basis of these data, we suppose that dimorphism of mucoraceous fungi depends on the physiological state of their spores, which is determined by biochemical processes proceeding in cells. Changes in the lipid composition, that is decrease in the degree of fatty acid unsaturation, content of storage and membrane lipids, as well as emergence and increase in the content of signal molecules and degradation products of lipidic nature in sporangiospores, together with the changes in elemental composition, accompany morphogenesis and are able to affect the subsequent development cycle upon spore germination.

Calcium is known to play an extremely important regulatory role in the processes of signal transduction, spore germination, growth, and morphogenesis; while it may be toxic for the cells, its concentration depends on functioning of a number of transporters and genes regulating calcium homeostasis [22–29]. Earlier studies of the elemental composition and the ratios of Ca/K and P/S in the cells of spore-forming (*Bacillus cereus*) and non-spore-forming bacteria (*Micrococcus luteus*), as well as in the yeast *Saccharomyces cerevisiae* [11], together with the results of the current work, showed that these indicators, together with the lipid characteristics, may be used in the development of criteria for evaluation of the physiological condition of microbial cells, including the viability of fungal sporangiospores. Moreover, this complex of data may be used to evaluate the capacity of mucoraceous fungi for dimorphism, in particular, in those species for which such ability has not been studied yet, for example, the species under study (*M. hiemalis*). The studies are to be continued since they broaden considerably the knowledge on abundance of the phenomenon of dimorphism and open its control in modern medicine, veterinary science, agriculture, and biotechnology to new perspectives.

## ACKNOWLEDGMENTS

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