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EXPERIMENTAL ARTICLES

Accumulation of Inorganic Polyphosphates in *Saccharomyces cerevisiae* under Nitrogen Deprivation: Stimulation by Magnesium Ions and Peculiarities of Localization

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Abstract—The yeast *Saccharomyces cerevisiae* was shown to have a high potential as a phosphate-accumulating organism under growth suppression by nitrogen limitation. The cells took up over 40% of phosphate from the medium containing 30 mM glucose and 5 mM potassium phosphate and over 80% of phosphate on addition of 5 mM magnesium sulfate. The major part of accumulated P_i was reserved as polyphosphates. The content of polyphosphates was ~57 and ~75% of the phosphate accumulated by the cells in the absence and presence of magnesium ions, respectively. The content of long-chain polyphosphates increased in the presence of magnesium ions, 5-fold for polymers with the average length of ~45 phosphate residues, 3.7-fold for polymers with the average chain length of ~75 residues, and more than 10-fold for polymers with the average chain length of ~200 residues. On the contrary, the content of polyphosphates with the average chain length of ~15 phosphate residues decreased threefold. According to the data of electron and confocal microscopy and X-ray microanalysis, the accumulated polyphosphates were localized in the cytoplasm and vacuoles. The cytoplasm of the cells accumulating polyphosphates in the presence of magnesium ions had numerous small phosphorus-containing inclusions; some of them were associated with large electron-transparent inclusions and the cytoplasmic membrane.

Keywords: *Saccharomyces cerevisiae*, inorganic polyphosphates, localization, nitrogen deprivation, cytosol, phosphate uptake, magnesium ions, DAPI.

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Inorganic polyphosphates (polyP) are the most widespread mineral phosphorous storage compounds in microorganisms [1–4]. Some data show the dependence of polyP accumulation on metal cations [1]; however, no special studies of this dependence have been carried out in the yeast *Saccharomyces cerevisiae*, where the metabolism of these polymers has been studied for a long time. Moreover, while polyP accumulation in many bacteria was shown to be stimulated under growth limitation conditions, including nitrogen deprivation [4], polyP accumulation in yeasts was studied under favorable growth conditions [1].

Microorganisms capable of efficient phosphate uptake are used for removal of excess phosphate from wastewater during the so-called enhanced biological phosphorus removal (EBPR) [5, 6]. Numerous bacterial species involved in such processes have been revealed and designated as phosphate-accumulating organisms (PAO) [7, 8]. The potential of yeasts as phosphate-accumulating organisms is still insufficiently studied, although the spores of an unidentified yeast species have been found in activated sludge [9]. The yeasts *Candida humicola* [10, 11], *Hansenula fabiani*, and *Hansenula anomala* [12], having a high ability to accumulate phosphate from the medium, were isolated from sewage waters. It was also shown that the ability of *S. cerevisiae* to accumulate phosphate from the medium could be increased by mutations in the genes encoding phosphate transport proteins [12].

The goal of the present work was to ascertain the conditions for the maximum uptake of phosphate (P_i) by the cells of *S. cerevisiae* under growth suppression by nitrogen deprivation and the peculiarities of polyP accumulation and localization in the presence of magnesium ions.

MATERIALS AND METHODS

The yeast *Saccharomyces cerevisiae* VKM Y-1173 from the All-Russian Collection of Microorganisms,

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Russian Academy of Sciences were grown on a shaker (120 rpm) at 29°C for 20 h up to the stationary growth phase in 200 ml of the medium containing the following (g/l): glucose, 20; yeast extract, 2; $(NH_4)_2SO_4$, 3; $MgSO_4 \cdot 7H_2O$, 0.7; $Ca(NO_3)_2$, 0.4; and K_2SO_4, 2.77. The medium contained 1 mM P_i as a yeast extract component. During the cultivation the pH value of the Reader medium changed from 6.0 to 3.5. After the cultivation, the cells were separated by centrifugation at 3000 *g* for 10 min, washed twice with distilled water, and used for polyphosphate extraction and P_i uptake experiments.

For determination of P_i uptake, the cells grown under P_i limitation (10 g of wet biomass per 1 l) were incubated under the same conditions in 25 ml of the medium containing glucose, 30 mM; KH_2PO_4, 5 mM; and $MgSO_4$, 5 mM. The initial pH value in these experiments was 5.5, while at the end of the experiment pH was 4.5 and 4.0 in the absence and presence of magnesium ions, respectively. The cases of using the media without magnesium sulfate or the media of other composition are specified in the table and figure captions. After incubation the cells were precipitated by centrifugation and used for extraction of phosphorous compounds.

P_i content in the culture liquid was determined according to [13], and glucose content was determined by the glucose oxidase method using a Diakon kit (Russia).

The following polyP fractions were extracted from the biomass: acid-soluble polyP1, salt-soluble polyP2, and alkali-soluble polyP3 and polyP4 [14]. Organic phosphorus-containing compounds were removed from the acid-soluble fraction polyP1 with activated carbon [14]. Residual biomass was extracted using distilled water, pH 7.0, for 20 h at 0°C; the precipitate was separated by centrifugation, and the supernatant was the fraction polyP5a. The precipitate was treated with 1 N $HClO_4$ at 90°C for 20 min; P_i released during the hydrolysis was assayed and its quantity was a measure of polyP content in the polyP5b fraction. In all fractions except for polyP5b, polyP was assayed by the content of labile phosphorus as described [14]. The content of P_i was assayed in advance. It should be noted that all P_i contained in the biomass was extracted into the polyP1 fraction, which is in agreement with the literature data [14]. The data of six experiments are presented; wet biomass value was used for calculations.

PolyP electrophoresis in PAAG was performed as described [15].

Staining with DAPI (4',6-diamidino-2-phenylindole) (Sigma, United States) and confocal microscopy were used for detection of polyphosphates in vivo.

The cells were stained in the standard PBS buffer, pH 7.4, at a dye concentration of 1 μg/ml and 30°C for 15 min. Then the cells were separated by centrifugation, resuspended in PBS with 1% agarose, and the drops on microscopic slides were covered with cover glasses and sealed with varnish to prevent drying. The preparations were examined in a Leica TSC SP5 laser scanning microscope with a PL APO 63x/1.40 oil λ (lambda blue) objective. Fluorescence was excited by a laser with $\lambda = 405$ nm. Emission was recorded in two spectral ranges: the first channel of 420–520 nm and the second channel of 540–640 nm. The image in transmitted light was registered by the DIC method.

Ultrathin sections were prepared as follows: biomass samples were fixed with 1.5% glutaraldehyde solution in 0.05 M cacodylate buffer, pH 7.2, at 4°C for 1 h. After three washings with the same buffer, the material was additionally fixed with 1% OsO_4 in the same buffer at 20°C for 3 h. After dehydration the material was embedded in Epon 812 and ultrathin sections were made with an LKB III Ultratome (Sweden). The sections were placed onto formvar-coated copper grids, contrasted with 3% uranyl acetate in 70% ethyl alcohol for 30 min, and then stained with led citrate according to [16]. A JEM-100B electron microscope (JEOL, Japan) was used for viewing.

Sections without additional treatment were used for X-ray microanalysis. The analysis was carried out in a JEM-100CXII electron microscope (Japan) equipped with an EM-ASID4 scanning device and an X-ray analyzer (Green Star) with an E5423 detector (Link-System, United Kingdom) at an 20000x magnification and a voltage of 80 kV.

RESULTS AND DISCUSSION

P_i uptake by *S. cerevisiae* cells under growth suppression by nutrient deprivation was studied under model conditions developed previously for various bacteria (Table 1) [17, 18]. It was shown that *S. cerevisiae* accumulated P_i as efficiently as the bacteria that have been already studied in this respect (Table 1) and the known bacteria from activated sludge [5–7]. Previously, the study of polyP metabolism in yeasts under growth conditions showed no efficient P_i uptake [1]. In contrast to bacteria, amino-acid mixture (casamino acids, Difco, United States) had a weak stimulatory effect on P_i accumulation by *S. cerevisiae* cells (Table 1). Neither *Acetobacter xylinum* nor members of the genus *Brevibacteria*, which have been used in our previous studies as model cultures for investigation of phosphate uptake, accumulated it in the absence of magnesium ions [17–19]. It is known that, at a high concentration, phosphate is transported into bacterial cells mainly via the Mg^{2+}–P_i symport system [20]. In yeasts, the magnesium-dependent phosphate transport system is unknown. While the yeast cells were able to take up P_i from the medium in the absence of magnesium ions (the presence of glucose was sufficient), the addition of 5 mM magnesium sulfate doubled this uptake

Table 1. P_i uptake from the medium by microorganisms of different systematic groups under growth-suppression conditions. Incubation time is 15 h for bacteria and 20 h for yeasts

Medium composition	Microorganism		
	Brevibacterium casei [17]	*Acetobacter xylinum* [18]	*Saccharomyces cerevisiae* VKM Y-1173
	P_i uptake from the medium, % of initial quantity		
5 mM KH_2PO_4, 5 mM $MgSO_4$	0	0	0
5 mM KH_2PO_4, 30 mM glucose	4	3	44 ± 6.9
5 mM KH_2PO_4, 5 mM $MgSO_4$, 30 mM glucose	4	50	84 ± 4.6
5 mM KH_2PO_4, 5 mM $MgSO_4$, 5 g per 1 l of amino acid mixture (Difco)	95	86	13 ± 1.0

(Table 1). Figure 1 shows the dynamics of decrease in the P_i and glucose concentrations in the incubation medium. The optical density of the culture did not change significantly, because the growth was initially suppressed by the absence of nitrogen source. Glucose uptake did not depend on the presence of magnesium ions, whereas phosphate uptake was stimulated by these ions throughout the experiment.

Polyphosphate content and chain length. After 20 h of incubation, the cells took up 200 and 420 μmol P/g of dry biomass in the absence and presence of Mg^{2+}, respectively. The major part of accumulated phosphate was stored as polyP (Table 2). Polyphosphate content was ~57 and ~75% of the phosphate accumulated by the cells in the absence and presence of magnesium ions, respectively. The P_i content in the cells was ~9 and 6% of the total ~9 consumed, respectively. In the presence of magnesium ions, polyP accumulation increased 2.5-fold (Table 2).

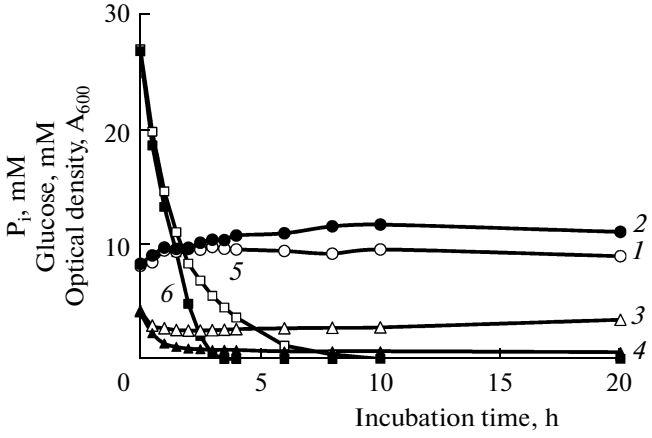

Fig. 1. Optical density of cell suspension and ambient concentrations of P_i and glucose during incubation of *Saccharomyces cerevisiae* VKM Y-1173 in the medium containing 30 mM glucose and 5 mM KH_2PO_4 (open symbols) and in the medium containing 30 mM glucose, 5 mM KH_2PO_4, and 5 mM $MgSO_4$ (shaded symbols): optical density (*1* and *2*), P_i concentration (*3* and *4*), and glucose concentration (*5* and *6*). The mean values of six experiments are presented; standard square deviation did not exceed 10%.

For comparison *Brevibacteria* are unable to accumulate polyP: their phosphorus reserve is P_i [17]; *A. xylinum* accumulates polyP under nitrogen deprivation in the presence of glucose and P_i in the presence of nitrogen sources and in the absence of glucose [18]. Glucose is required for polyP synthesis since P_i transport and formation of phosphoester bonds in polyP require energy.

In *S. cerevisiae* polyP is represented by various fractions differing both in chain length and in metabolic peculiarities [1, 14, 21]. It was shown that an increase in the total polyP content in the presence of Mg^{2+} was coupled to an increase in the content of polyP2, polyP3, polyP5a, and polyP5b, while the content of polyP1 even decreased (Table 2). PAGE electrophoresis (Fig. 2) showed that the average polyP chain length of the fractions was similar to that observed during growth in complete media [21] and did not change in the presence of Mg^{2+} (Table 2). In the presence of magnesium ions, the content of long-chain polyphosphates increased: 5-fold for polymers with an average length of ~45 phosphate residues, 3.7-fold for polymers with an average chain length of ~75 residues, and more than 10-fold for polymers with an average chain length of ~200 residues. The content of polyphosphates with the average chain length of ~15 phosphate residues, on the contrary, decreased threefold under these conditions (Table 2).

PolyP localization according to electron microscopy and X-ray microanalysis. Electron-dense inclusions in polyP-accumulating yeast cells have been known for some time [22]. In our experiments the cells that had accumulated polyP contained numerous small electron-dense inclusions in the cytoplasm and vacuoles and their number in the cytoplasm increased significantly in the presence of Mg^{2+} (Fig. 3b and 3c). The cells grown under phosphate deprivation had no such inclusions (Fig. 3a). The peculiar feature of localization of these inclusions in the presence of Mg^{2+} was their presence close to the cytoplasmic membrane and in association with large electron-transparent inclu-

Table 2. The content of P_i and polyP of different fractions (μmol/g of wet biomass) with indication of the average chain length (~*n*) in the cells of *S. cerevisiae* VKM Y-1173: cells grown on P_i deficient medium up to the stationary growth phase (−P); cells incubated for 20 h on the medium with 5 mM KH_2PO_4 and 30 mM glucose (+P); and cells incubated for 20 h with 5 mM KH_2PO_4, 3 mM glucose, and 5 mM $MgSO_4$ (+P + Mg)

Fraction	Cultivation and incubation conditions				
	−P	+P		+P + Mg	
	μmol/g of wet biomass	μmol/g of wet biomass	~*n*	μmol/g of wet biomass	~*n*
P_i	7.7 ± 0.6	18 ± 2.0		26 ± 4.6	
polyP1	0.4 ± 0.1	74 ± 4.5	15	23 ± 1.0	15
polyP2	0.5 ± 0.1	22 ± 1.4	45	110 ± 8.2	45
polyP3	1.5 ± 0.6	26 ± 2.4	75	96 ± 13	75
polyP4	0.9 ± 0.4	3.6 ± 0.4	200	21 ± 2.1	200
polyP5a	0.2 ± 0.2	0.5 ± 0.2	200	23 ± 1.5	200
polyP5b	0.5 ± 0.1	1.3 ± 0.5		17 ± 0.5	
Σ polyP	4.0 ± 1.1	126 ± 9.8		316 ± 28	

sions (Fig. 3c). X-ray microanalysis showed that these small inclusions contained phosphorus (Fig. 4). Taking into account the extraction data, these inclusions may be supposed to contain polyP. The granules associated with electron-transparent inclusions contained more phosphorus than did those scattered over the cytoplasm (Fig. 4). X-ray microanalysis revealed no cations associated with these structures. Hence, the role of magnesium ions in phosphate accumulation by yeast cells and in polyP synthesis requires further investigation. It should be noted that the only currently known yeast polyphosphate kinase is stimulated by manganese ions and, to a lesser extent, by magnesium ions [23].

Fluorescence microscopy. DAPI fluorescent dye is used for staining DNA, polyP, and lipid inclusions in

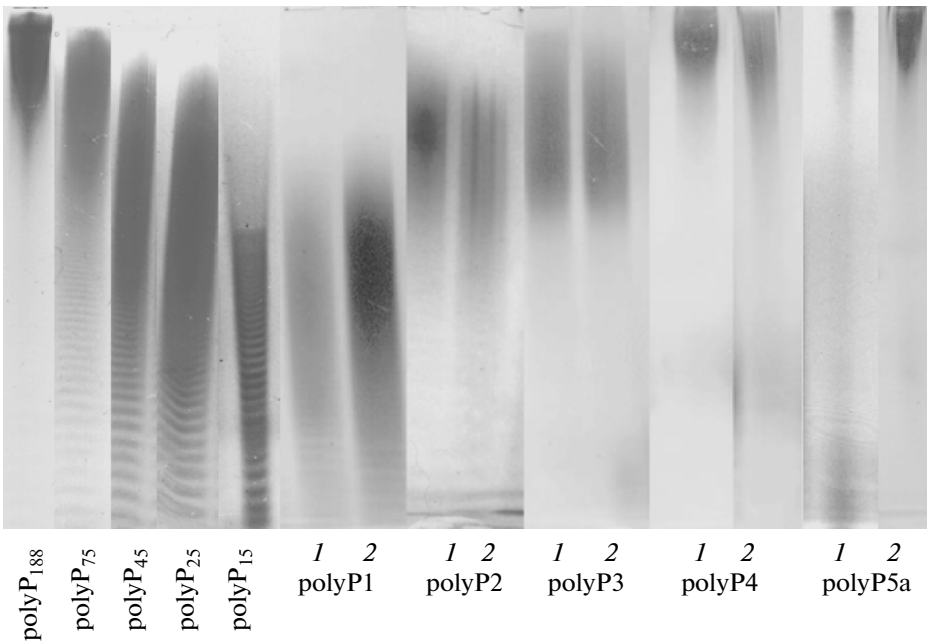

Fig. 2. Electrophoregrams of polyP from different fractions in 20% PAAG in the presence of 7 M urea. PolyP extracted from the cells incubated in the medium containing 30 mM glucose and 5 mM KH_2PO_4 (*1*) and polyP extracted from the cells incubated in the medium containing 30 mM glucose, 5 mM KH_2PO_4, and 5 mM $MgSO_4$ (*2*). Incubation time, 20 h. $PolyP_{188}$, $polyP_{75}$, $polyP_{45}$, $polyP_{25}$, and $polyP_{15}$ are commercial polyphosphates with average chain lengths of 188, 75, 45, 25, and 15 phosphate residues, respectively, used as labels.

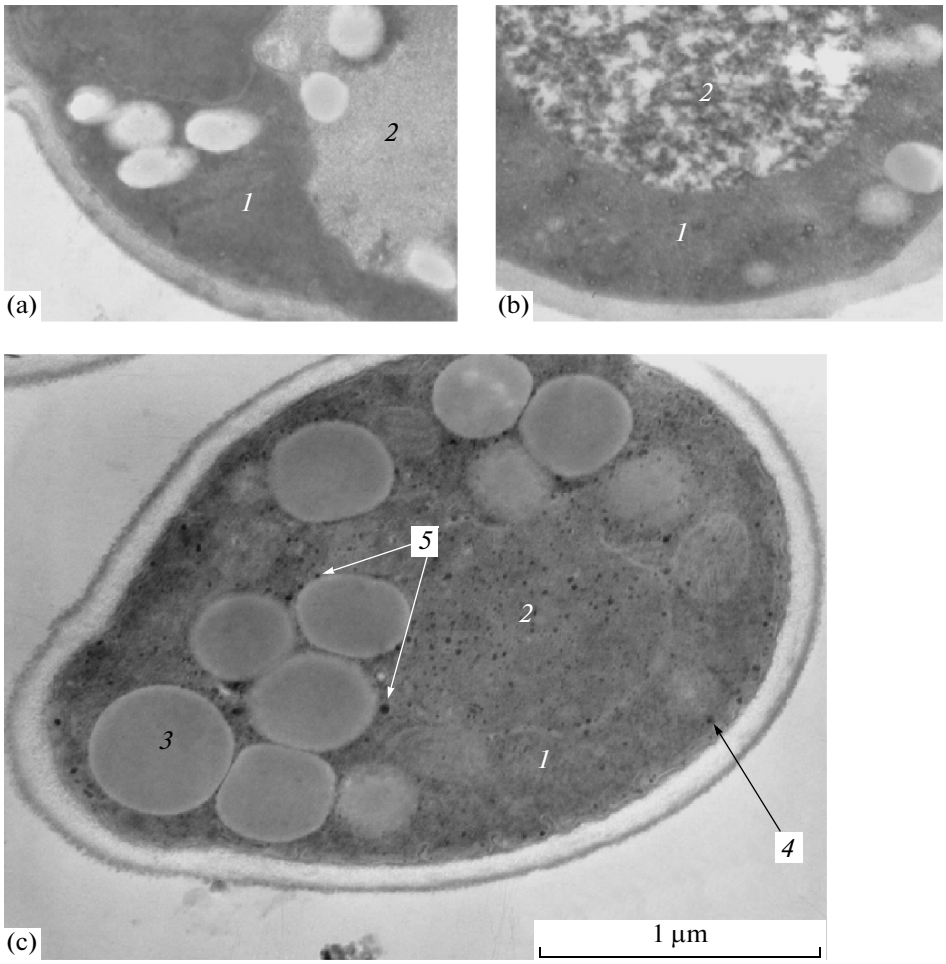

Fig. 3. Ultrathin sections of *S. cerevisiae* cells: control cells grown on P_i-deficient medium up to the stationary growth stage (a), cells after incubation in the medium containing 30 mM glucose and 5 mM KH_2PO_4 for 20 h (b), and cells after incubation in the medium containing 30 mM glucose, 5 mM KH_2PO_4, and 5 mM $MgSO_4$ for 20 h (c). Cytoplasm (*1*), vacuole (*2*), lipid inclusions (*3*), electron-dense granules close to the cytoplasmic membrane (*4*), and electron-dense granules associated with lipid inclusions (*5*).

the cells [24–26]. The DAPI–DNA and DAPI–polyP complexes fluoresce in the blue and yellow–orange spectral regions, respectively, while the fluorescence of the DAPI complexes with lipids is pale-yellow and fades within a few seconds [24]. Confocal microscopy showed that the preparations of unfixed *S. cerevisiae* cells, which had accumulated polyP in the presence of Mg^{2+} ions, contained inclusions with bright-yellow fluorescence (Fig. 5). This fluorescence was so intense that it masked the blue glow of the nuclei. These inclusions were similar in size and position within a cell to the electron-transparent inclusions found on thin sections. No data on DAPI staining of glycogen inclusions was found in the literature, while the fluorescence of DAPI-stained lipid inclusions has been observed [24]. We assume that the bright-yellow fluorescence of inclusions results from the fact that these inclusions are associated with smaller polyP-containing granules, as was shown by electron microscopy and X-ray microanalysis. No inclusions with DAPI-induced fluorescence were found in the control cells incubated in the presence of glucose and magnesium sulfate but without phosphate. Only the nuclei fluoresced in the original cells, which were grown under P_i deprivation and contained little polyP (Table 2, first column) (Fig. 5). The fluorescence of the nuclei was blue, in agreement with the literature data [26]. The combined data of electron and confocal microscopy and X-ray microanalysis suggest that the cytoplasm of *S. cerevisiae* during phosphate uptake under the conditions of growth suppression and in the presence of Mg^{2+} ions contains polyP not only as small inclusions in the cytoplasm and vacuoles, but also as associations with larger inclusions of supposedly lipid nature. Phosphorus-containing inclusions, which were located separately in the vacuoles and cytoplasm, were small in size and, therefore, could not be revealed by fluorescence microscopy. It should be noted that the method of DAPI staining, although specific and demonstrative, does not reveal all the polyP present in yeast cells. The data obtained are in agreement with

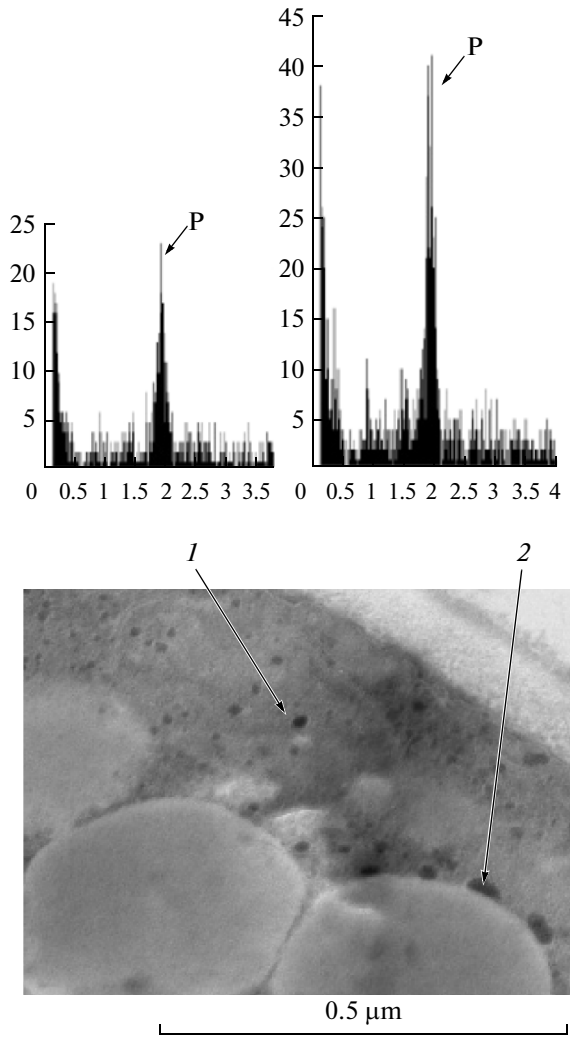

Fig. 4. Ultrathin section and X-ray microanalysis of *S. cerevisiae* cells that have accumulated polyP in the presence of Mg ions: small electron-dense granules in the cytoplasm (*1*) and small electron-dense granules associated with electron-clear inclusions (*2*).

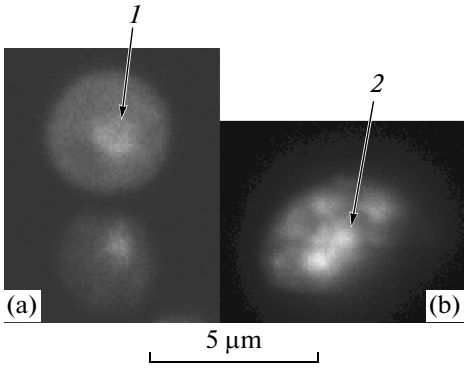

Fig. 5. DAPI-stained *S. cerevisiae* cells: control cells grown on P_i deficient medium up to the stationary growth stage (a) and cells after 20-h incubation in the medium containing 30 mM glucose, 5 mM KH_2PO_4, and 5 mM $MgSO_4$ (b). Nucleus (blue fluorescence) (*1*) and inclusions (bright yellow fluorescence) (*2*).

our concept of multiple localization of polyP in yeast cells based on the investigation of subcellular fractions [15].

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