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EXPERIMENTAL ARTICLES

Effect of Cycloheximide, Iodoacetamide, and Antimycin A on Inorganic Phosphate Synthesis in *Saccharomyces cerevisiae* VKM Y-1173

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Abstract—The effect of inhibitors of protein synthesis (cycloheximide, CHI), glycolysis (iodoacetamide, IAA), and oxidative phosphorylation (antimycin A, ANM) on inorganic phosphate (polyP) synthesis during the first 0.5 h of their hypercompensation in *Saccharomyces cerevisiae* VKM Y-1173 grown on 2% glucose-containing media at low (hypoxia) or high aeration rates or in the presence of 1 vol % ethanol under high aeration conditions was studied. PolyP accumulation was highest in the medium with glucose under hypoxia; lower, with glucose at high aeration; and lowest, in the medium with ethanol. CHI had a small effect on the total polyP level but significantly stimulated ATP accumulation, irrespective of the culture growth conditions. The low-polymer acid-soluble polyP1 were synthesized most actively by the cells grown on glucose under hypoxia, alkali-soluble polyP3 were synthesized at enhanced aeration, and the most high-molecular fraction, polyP5, was actively accumulated along with polyP3 at cultivation on ethanol. Regardless of the growth conditions, CHI inhibited accumulation of polyP4, the synthesis of which is associated with the synthesis of mannoproteins. IAA and ANM largely inhibited synthesis of all fractions at yeast growth under hypoxia and on ethanol, respectively. The results as a whole demonstrate the dependence of polyP formation on the main energy-generating cell processes and, at the same time, the absence of direct dependence of their synthesis on ATP concentration in *Saccharomyces cerevisiae* VKM Y-1173.

Key words: inorganic polyphosphates, carbon source, aeration, hypercompensation, inhibitors, *Saccharomyces cerevisiae*.

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Inorganic polyphosphates (polyPs), linear polymers composed of orthophosphate residues linked by high-energy phosphoanhydride bonds, were found in all studied organisms from bacteria to mammals [1–5].

Lately, the compounds attract increased attention due to new facts indicating polyP involvement in many metabolic processes. Apart from their function of accumulation of phosphorus and energy which are easily utilized under conditions of their depletion, polyPs are important in regulation of various enzymes and gene expression, in the maintenance of cation levels and pH homeostasis, and organization of the transport channels and cell wall structure [4, 5].

Chemical fractionation of yeast cells results in isolation of five polyP fractions different in cell localization and chain length. The number of orthophosphate residues in a polyP molecule may vary depending on yeast growth phase and culturing conditions.

^{31}P NMR spectroscopy demonstrated that the lowest molecular weight fraction with the number of orthophosphate residues in the chain ($\tilde{n}$) from 3 to 28 is an acid-soluble polyP1 fraction. Salt-soluble polyP2 fraction contains polymers of $\tilde{n}$ = 20–30. The alkali-soluble fraction (pH 9–10) polyP3 is a polymer of $\tilde{n}$ = 32–40, while another alkali-soluble one (pH 12), polyP4, of $\tilde{n}$ = 50–60 [6, 7]. Finally, the acid- and alkali-insoluble fraction polyP5 with the highest molecular weight contains $\tilde{n} > 200$ orthophosphate residues [8].

While polyP degradation pathways and the related enzymes of *S. cerevisiae* have been studied rather well [4, 5, 9], many aspects of polyP synthesis are still an open issue. In bacteria, polyP synthesis is carried out at the expense of the ATP terminal phosphate in the presence of polyphosphate kinase (EC 2.7.4.1) [3–5]. Recently, a similar enzyme was discovered in *Candida humicola* G1 [10]. Searching for such enzyme in *S. cerevisiae* has not been successful [11]. On the other hand, it has been demonstrated that accumulation of the polyP4 fraction localized beyond the cytoplasmic membrane in the cell wall correlates with accumulation of the cell wall mannoproteins and is mediated by dolichyl-diphosphate phosphotransferase (EC

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2.7.4.20) [12]. Recently, it was shown that vacuolar transport chaperones phm1–phm4, responsible for vacuolar H^+-ATPase localization, may be involved in the synthesis of vacuole-localized polyP [13].

In yeast, the polyP4 fraction content does not exceed 20% of the total cell polyP, while the amount of vacuolar polyP in *S. cerevisiae* VKM Y-1173 is not more than 14–18% to the total polyP content [14]. Therefore, the pathways of formation of the main polyP pool in *S. cerevisiae* are still to be elucidated.

Significant effect on polyP accumulation is produced by yeast growth conditions, including P_i content in the medium, carbon source, and aeration [15]. It has been shown that polyP accumulation is most efficient in the middle of the exponential phase at cultivation on glucose-containing medium under hypoxia and is less pronounced on ethanol [15, 16].

PolyPs are actively involved in energy flows as additional energy sources under conditions of active yeast growth [15]. The relations between polyP synthesis and energetic processes characteristic of the specific growth conditions remain poorly investigated. An inhibitor of oxidative phosphorylation, antimycin A, was shown to suppress polyP synthesis in *S. cerevisiae* Delft 1 grown on ethanol [16], while iodoacetamide, a glycolysis inhibitor, had almost no effect on polyP accumulation in glucose-grown cells [17]. The latter data were obtained after cell transfer from phosphate-deficient to P_i-containing medium (polyP hypercompensation) at a long, 2-h exposure. However, it was recently established that it is more appropriate to assay polyP synthesis at hypercompensation after a short-term exposure (not more than 30–40 min), as later either the polyP synthesis rate decreases or processes of polyP consumption start to take over [8].

In this relation, the aim of the work was to study the effects of inhibitors of the major metabolic processes on polyP synthesis under conditions of short-term hypercompensation in *S. cerevisiae* VKM Y-1173 grown on media with various carbon sources and at different degrees of aeration.

MATERIALS AND METHODS

Yeast strain *Saccharomyces cerevisiae* VKM Y-1173 was the object of the study. The cells were grown on a shaker at 29°C in flasks with Rider medium [18] containing 2% glucose as a carbon source, at low, <0.1 mmol O_2/l min (hypoxia), or high, 0.56 mmol O_2/l min aeration, and at high aeration degree with 1 vol % ethanol [15].

PolyP fractions were obtained according to Langen and Lis [6] by successive extraction with acid, salt, and alkali solutions in cold. The following polyP fractions were isolated, acid-soluble (polyP1), salt-soluble (polyP2), and two alkali-soluble fractions, at pH 9–10 (polyP3) and pH 12 (polyP4). The content of the polyP5 fraction soluble in neither acid, nor alkali solutions, was evaluated from the orthophosphate formed upon treatment of the remaining biomass with 0.5 M $HClO_4$ at 90°C twice for 20 min.

Conditions of sample preparation and separation of the polyP fractions by electrophoresis in polyacrylamide gel were as described in [15].

ATP extraction and determination was performed with the luciferin–luciferase preparation (Sigma, United States) on a 1250 luminometer (LKB, Sweden) as was previously described [19].

Determination of phosphorus compounds, P_i, glucose, optical density (OD), and yeast dry biomass was performed according to known methods [6].

The data of three biological experiments statistically processed [20] are presented.

RESULTS AND DISCUSSION

Hypercompensation conditions are used to study polyP synthesis in yeast. For this purpose, after a certain period of P_i deficiency, the cells are transferred to a P_i-containing medium [8, 18].

Earlier, we have demonstrated that all polyP fractions are most efficiently synthesized during the first 0.5 h of the experiment, and later, with different intensities in different fractions, the processes of polyP consumption are probably activated, resulting in decreased accumulation [8]. Therefore, in the present work, the phase of active polyP synthesis was studied at 0.5-h exposure under hypercompensation conditions with 2% glucose at hypoxia or high aeration rate and with 1 vol % ethanol at high aeration [15].

Data on P_i, ATP, and total polyP content in the middle of the exponential growth phase of *S. cerevisiae* VKM Y-1173 on full Rider medium (0 h, +P), after the subsequent 7-h starvation (7 h, –P), and during the first 0.5 h of hypercompensation (7.5 h, +P, hypercompensation) are presented in Table 1.

As follows from the data, cells grown on ethanol contained less P_i than those grown on glucose independently of the aeration degree. On the contrary, ATP content was almost three times higher in the cells grown on ethanol than on glucose. PolyP content in the cells depended upon carbon source as well as aeration; however, regardless of it, it decreases in the row glucose (hypoxia) ⟶ glucose (aeration) ⟶ ethanol (aeration).

During P_i starvation, yeast cells continued active growth (Table 1, OD). Meanwhile, cellular P_i content decreased almost three times, and polyP amount decreased depending on the cultivation conditions, that is, on glucose-containing medium under hypoxia conditions, by a factor of 18; on glucose-containing medium at aeration, by a factor of 8; and on ethanol, by a factor of 6.

Unlike P_i and polyP, ATP content upon growth on glucose under the experimental conditions even increased somewhat, and upon growth on ethanol, it decreased, yet remaining relatively high. The data may

Table 1. Growth and P_i, ATP, and polyP content of *S. cerevisiae* VKM Y-1173 grown on glucose or ethanol, μmol/g dry weight

Growth, h	Cultivation conditions	2% glucose, hypoxia				2% glucose, high aeration				% ethanol, high aeration			
		OD_{530}	P_i	ATP	polyP	OD_{530}	P_i	ATP	polyP	OD_{530}	P_i	ATP	polyP
0	+P	0.72	106.6 ± 8.1	3.4 ± 0.21	543 ± 47	0.67	104.2 ± 8.9	3.2 ± 0.2	508.7 ± 40	0.49	82.9 ± 8.2	9.1 ± 0.9	309.6 ± 23
7.0	−P	2.09	33.9 ± 3.3	4.3 ± 0.28	31.8 ± 3.3	2.15	35.1 ± 5.1	4.0 ± 0.24	65.2 ± 7.9	1.53	24.5 ± 3.3	6.4 ± 0.4	48.5 ± 6.1
7.5	+P, hypercompensation	2.28	101.5 ± 7.4	56 ± 0.3	1024.5 ± 74	2.30	107.2 ± 9.5	5.8 ± 0.22	849.0 ± 61	1.71	82.5 ± 7.2	71.3 ± 0.6	719.3 ± 51

Table 2. Accumulation of individual polyP fractions in *S. cerevisiae* VKM Y-1173 under hypercompensation conditions, μmol P/g dry weight

Culturing conditions	PolyP1	PolyP2	PolyP3	PolyP4	PolyP
2% glucose, hypoxia	471.9 ± 33.7	99.5 ± 7.8	252.2 ± 21.4	84.5 ± 6.3	116.4 ± 8.3
2% glucose, high aeration	269.2 ± 18.3	98.2 ± 8.1	316.7 ± 19.8	103.3 ± 9.2	61.6 ± 5.7
1% ethanol, high aeration	157.5 ± 10.3	70.4 ± 7.8	269.7 ± 17.5	85 ± 7.1	136.7 ± 7.9

testify to involvement of polyP in the maintenance of high ATP level in yeast cells under conditions of phosphate depletion in the medium [21].

After cell transfer to a P_i-containing medium, during the first 0.5 h, culture growth went on (Table 1, OD). The cell level of P_i was restored almost completely and ATP content increased somewhat. Irrespective of growth conditions, polyP amount increased sharply in comparison to the initial culture (0 h, +P); more precisely, at growth on glucose and hypoxia, it almost doubled; at growth on glucose and high aeration, it increased by 1.7 times; and at growth on ethanol, by 2.3 times. Therefore, extra synthesis of polyP (hypercompensation) occurred independently of carbon source or aeration rate. However, the hypercompensation phenomenon apparently cannot does not apply to all yeast-like organisms as it was not detected, for example, in *Candida humicola* [10]. Accepting the data collected after phosphorus starvation (Table 1, 7.0 h, −P) as initial conditions, the maximum polyP synthesis rate is observed in the culture grown on glucose under hypoxia (33.1 μmol P_i min^{-1} mg^{-1} dry biomass). At higher aeration, polyP synthesis rate decreased significantly (26.1 μmol P_i min^{-1} mg^{-1} dry biomass) and practically did not change for yeast grown on ethanol (22.4 μmol P_i min^{-1} mg^{-1} dry biomass), which suggests a possible evolutionary bond between polyP exchange and glycolysis [4].

The data presented in Table 1 (7.5 h, +P, hypercompensation) are used as the control ones in the experiments with inhibitors of the major metabolic processes, an inhibitor of protein synthesis at translation level, cycloheximide (CHI); an inhibitor of glycolysis, iodoacetamide (IAA); and an inhibitor of oxidative phosphorylation at the level of the respiratory chain, antimycin A (ANM).

At the indicated concentrations, all these inhibitors suppressed culture growth (data not shown).

As follows from Fig. 1, CHI effect independently of the cultivation conditions led to a slight (by 3–9%) decrease of cellular P_i and a moderate (by 7–20%) increase in the total polyP level. Under the same conditions, ATP content increased sharply, by a factor of 2 upon growth on glucose at hypoxia; 4.4 times, upon growth on glucose at high aeration; and 2.8 times, when grown on ethanol. This significant increase in ATP level was probably due to termination of one of the most energy-consuming processes—namely, protein synthesis. It may also be noted that increase of the ATP content in yeast depended not only on the carbon source, but on aeration intensity as well.

IAA activity resulted in some P_i decrease (by 10–20%) and a sharp decrease in ATP (by 85–95%) and polyP (by 65–85%) content when grown on glucose independently of medium aeration conditions and to a significantly lesser degree upon growth on ethanol (ATP, by 65%, and polyP, by 35%).

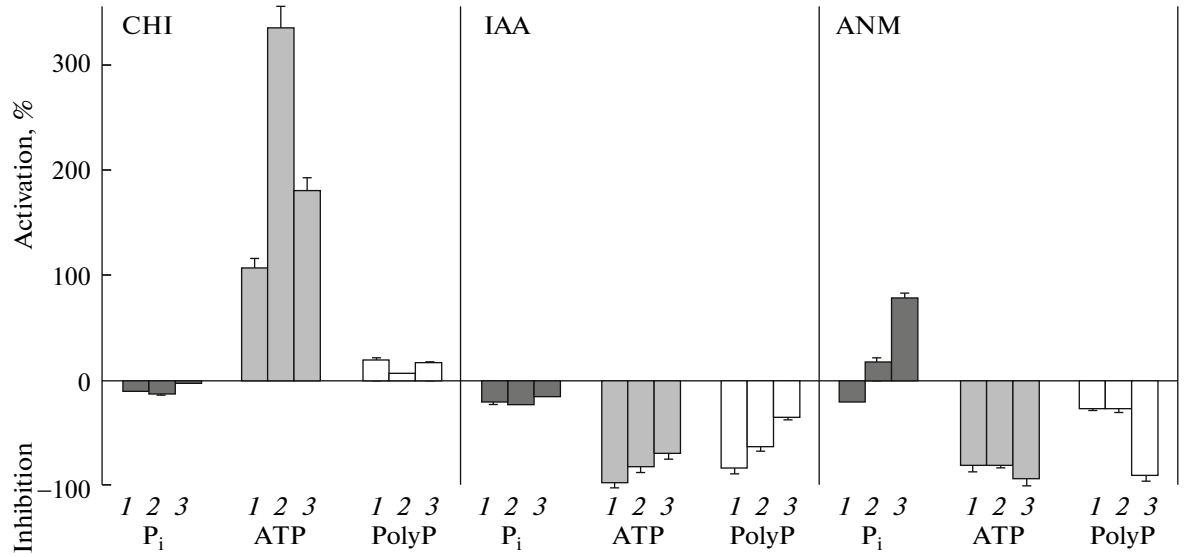

Fig. 1. Effect of the inhibitors on P_i, ATP, and polyP content in yeast cells after 0.5-h cultivation under hypercompensation conditions on glucose at hypoxia (*1*), on glucose at high aeration (*2*), and on ethanol (*3*), expressed as a percent of the control values presented in Table 1.

Treatment with ANM decreased P_i level in the cells grown on glucose under hypoxia and increased its content by 20% on the same carbon source at intense aeration. In yeast grown on ethanol the inhibitor promoted P_i increase even further (by 70%). ANM inhibited ATP formation by 70–80% in yeast grown on glucose and by over 90%, in those grown on ethanol. Under these conditions, polyP synthesis was suppressed by 30–35% in the cells grown on glucose and by 90% in those grown on ethanol.

Therefore, both IAA and ANM decreased ATP content significantly, as well as polyP synthesis; notably, IAA decreased ATP and polyP levels to a greater extent in the yeast grown on glucose under hypoxia, while ANM activity resulted in a more significant drop of their levels in the cells grown on ethanol.

Study of dozens of strains of the *Saccharomyces* complex has shown that most of them are able to grow on glucose or fructose under hypoxia conditions (Crabtree effect) and to form respiratory-deficient mitochondrial petite mutants. However, a number of organisms have demonstrated a decreased Crabtree effect exhibiting higher growth rate in media with aeration than under hypoxia [22].

Earlier, we have demonstrated that *S. cerevisiae* VKM Y-1173 cells grew on glucose-containing medium with aeration much faster ($\mu = 0.52\ h^{-1}$) and with higher biomass yield than under hypoxia conditions ($\mu = 0.38\ h^{-1}$) [15]. Therefore, the strain may be considered an organism with a decreased Crabtree effect. In contrast to *S. cerevisiae* BII, exhibiting very low total cytochromes concentration when grown on glucose, with cytochrome *a* below the detection limit [23], in *S. cerevisiae* VKM Y-1173 grown on glucose under hypoxia cytochrome levels remained sufficiently high, decreasing only by 50% compared to the cells grown on ethanol [15].

It is probable that these properties of the strain under study determine the effect produced by IAA on the cells grown on ethanol and by ANM, on the cells grown on glucose.

The degree of ANM influence on yeast growth is presently suggested as an important test in difficult taxonomic problems related to the *Saccharomyces* complex [22]. Besides, in *S. cerevisiae* cultures grown on glucose at high aeration, overproduction of Hap4p, a positive transcription regulator of the genes responsible for respiratory metabolism, was observed [24].

Therefore, when grown on glucose with high oxygen content in the medium, *S. cerevisiae* undergo considerable metabolic changes preparing the transformation from fermentative to respiratory life style.

PolyPs in *S. cerevisiae* VKM Y-1173 are known to be represented by at least five fractions localized in various compartments of the cell. As has already been mentioned, these fractions differ by extraction procedure and chain length.

Data on the content of various fractions under conditions of their active synthesis at hypercompensation are presented in Table 2. Clearly, in yeast grown on glucose under hypoxia conditions, polyP1 accumulated most efficiently during the first 0.5 h; at higher aeration on the same carbon source, the polyP3 fraction was synthesized most actively; and in the cells cultured on ethanol, the polyP5 fraction increased significantly along with polyP3.

In order to investigate how the shift in energy exchange type may influence the synthesis of individ-

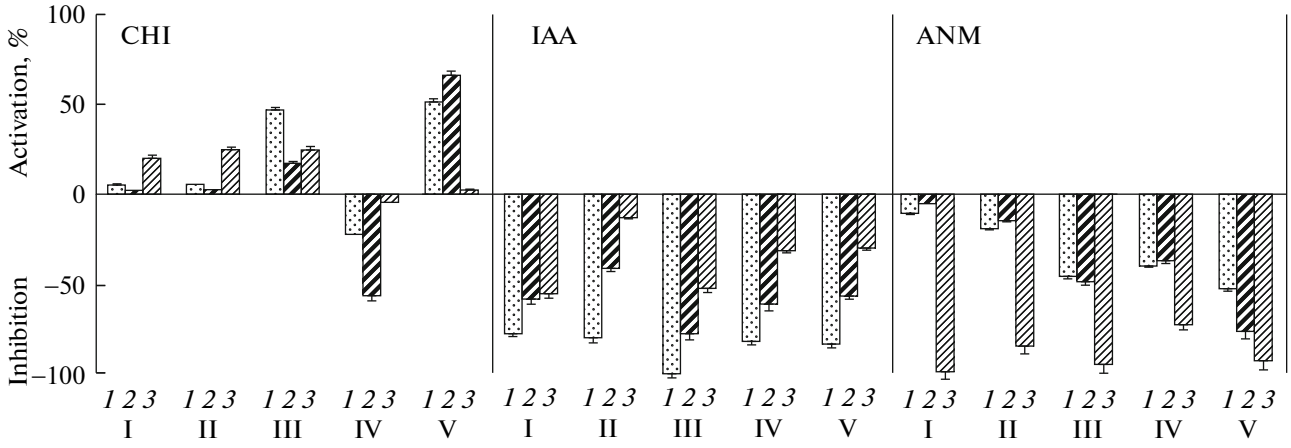

Fig. 2. Effect of the inhibitors on synthesis of polyP fractions in yeast cells after 0.5-h cultivation under hypercompensation conditions on glucose at hypoxia (*1*), on glucose at high aeration (*2*), and on ethanol (*3*), expressed as a percent of the control values presented in Table 1; I, II, II, IV, and V stands for polyP1, polyP2, polyP3, polyP4, and polyP5 fraction, respectively.

ual fractions, the effect of the relevant inhibitors was studied. The data are presented on Fig. 2. The data presented in Table 2 were considered the control data. As follows from Fig. 2, CHI resulted in accumulation of almost all polyP fractions irrespectively of the carbon source and the aeration intensity. Only polyP4 fraction synthesis was more or less suppressed under treatment with the inhibitor, thus supporting the data on the interrelation between the synthesis of this fraction and protein synthesis [17]. The polyP4 fraction is localized in the cell wall, and the mechanism of its synthesis is definitely connected to the synthesis of cell wall mannoproteins [12]. This is the reason inhibition of mannoprotein synthesis by CHI hampers polyP4 accumulation as well.

Activity of a glycolysis inhibitor, IAA, suppressed accumulation of all polyP fractions to the largest degree (by 75–100%) in yeast grown on glucose under hypoxia. Its activity decreased (by 40–55%) with an increase in the aeration rate. IAA suppressed accumulation of polyP fractions to the lowest extent (by 15–55%) in the cells cultivated on ethanol.

An inhibitor of oxidative phosphorylation, ANM, in yeast grown on glucose uniformly suppressed polyP1, 2, 3, and 4 synthesis independently of the aeration degree. PolyP5 synthesis was more hampered in the cells grown on glucose with high aeration and significantly hampered (by 90%) in ethanol-grown cells, which supports the dependence of accumulation of polyP5 fractions on the process of oxidative phosphorylation [8]. In yeast grown on ethanol, synthesis of all polyP fractions and especially polyP1 and polyP3 was suppressed by 70–100%.

PolyP fractions study with PAGE revealed that the inhibitors used in the present work at culturing both on glucose (Fig. 3) and ethanol (data not shown) had practically no effect on the polymerization degree of the fractions. These results support the separative nature of enzymatic systems involved in the synthesis of different polyP fractions localized in different cellular compartments [1, 4, 5].

As was already mentioned, in *S. cerevisiae* synthesis of polyP localized in the cell wall is connected to mannoproteins synthesis. Data exist suggesting that vacuolar polyP synthesis is under control of the *PHM1–PHM4* genes similar to the *VTC1–VTC4* genes of transport chaperones responsible for localization of vacuolar H^+-ATPase [13, 25].

The presented data evidence that polyP synthesis in *S. cerevisiae* VKM Y-1173 is in direct connection to the energy-producing processes of the cell, either glycolysis in yeast grown on glucose, or oxidative phosphorylation, in yeast grown on ethanol.

It is also possible that yeast, similar to some fungi, may perform polyP synthesis employing 1,3-diphosphoglycerate polyP phosphotransferase (EC 2.7.4.17) elongating the polyP chain bypassing ATP [1, 4, 5]. The data obtained in the present work do not prove the direct connection between polyP biosynthesis and ATP concentration in cells. This is most likely due to the absence in *S. cerevisiae* of ATP polyP phosphotransferase, an enzyme using terminal ATP phosphate in polyP synthesis [11]. However, ATP may influence polyP synthesis indirectly, in particular via the activity of electrogenic proton pumps, membrane ATPases, which generate hydrogen ions gradient $\Delta\mu H^+$ used in many energy-dependent transport processes. The possibility of polyP synthesis without direct participation of ATP is debated in the literature [13] and has been somewhat supported by the effect produced by bafilomycin, an inhibitor of vacuolar H^+-ATPase, on polyP accumulation in *S. cerevisiae* VKM Y-1173 [17].

Searching for polyP synthesis pathways in various compartments of yeast cells is a topical issue, and the study of interrelations between the polymer synthesis

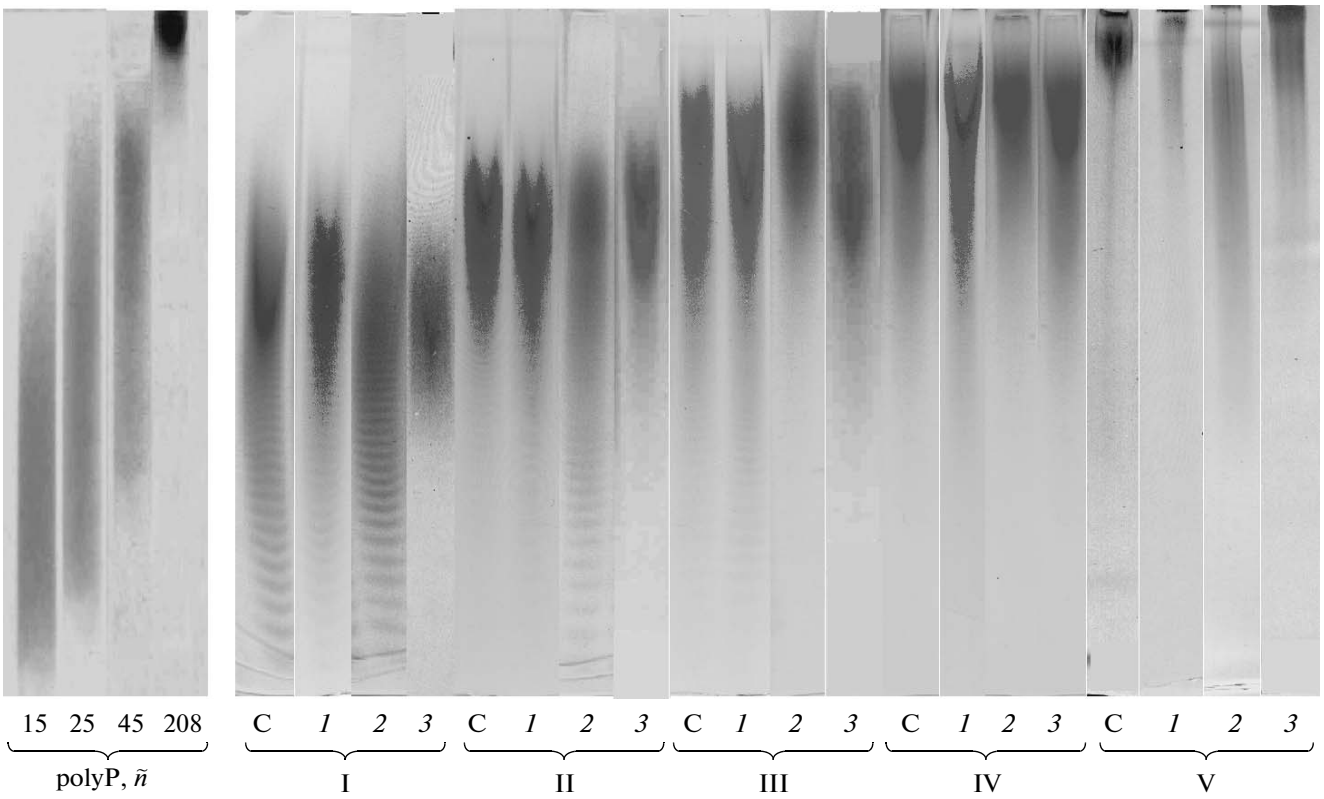

Fig. 3. Electrophoresis of polyP fractions synthesized by *S. cerevisiae* cells after 0.5-h cultivation under hypercompensation conditions on glucose at high aeration. C control yeast cells, treated with CHI (*1*), with IAA (*2*), and with ANM (*3*); I, II, II, IV, and V stands for polyP1, polyP2, polyP3, polyP4, and polyP5 fraction, respectively; *ñ* stands for polymerization degree of the standard polyP preparations.

and the energetic state of the membranes is also promising.

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