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Specific Features of Changes in Levels of Endogenous Respiration Substrates in *Saccharomyces cerevisiae* Cells at Low Temperature

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Abstract—The rate of endogenous respiration of *Saccharomyces cerevisiae* cells incubated at 0°C under aerobic conditions in the absence of exogenous substrates decreased exponentially with a half-period of about 5 h when measured at 30°C. This was associated with an indirectly shown decrease in the level of oxaloacetate in the mitochondria *in situ*. The initial concentration of oxaloacetate significantly decreased the activity of succinate dehydrogenase. The rate of cell respiration in the presence of acetate and other exogenous substrates producing acetyl-CoA in mitochondria also decreased, whereas the respiration rate on succinate increased. These changes were accompanied by an at least threefold increase in the L-malate concentration in the cells within 24 h. It is suggested that the increase in the L-malate level in the cells and the concurrent decrease in the oxaloacetate level in the mitochondria should be associated with a deceleration at 0°C of the transport of endogenous respiration substrates from the cytosol into the mitochondria. This deceleration is likely to be caused by a high Arrhenius activation energy specific for transporters. The physiological significance of L-malate in regulation of the *S. cerevisiae* cell respiration is discussed.

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Key words: endogenous cell respiration, low temperature, L-malate, oxaloacetate, malonate, transporters, *Saccharomyces cerevisiae*

Endogenous respiration of *Saccharomyces cerevisiae* cells is responsible for cell viability in the absence of exogenous substrates. The respiratory function of mitochondria and metabolism of reserve carbohydrates are interrelated [1-5]. The reserve carbohydrates, such as the polysaccharide glycogen and the disaccharide trehalose, are main sources of endogenous substrates for mitochondria. Accumulation (biosynthesis) of these reserve compounds is activated on a decrease in the level of endogenous substrates [4, 6], whereas their utilization increases upon exhaustion of key endogenous metabolites, e.g., glucose-6-phosphate [7]. Metabolism of reserve carbohydrates is a complicated system due to intricate interactions between the accumulation [7, 8] and utilization in the cell of these carbohydrates under different stress conditions [9-11].

Under aerobic conditions, glycogen phosphorylase is activated in the cells immediately upon exhaustion of exogenous glucose [12]. The generated glucose-1-phosphate is converted during glycolysis to phosphoenolpyruvate (Scheme). Pyruvate produced during the pyruvate kinase-catalyzed reaction is a key metabolite providing for the endogenous respiration of cells. Some pyruvate is oxidized in mitochondria with production of acetyl-CoA, which is one of two substrates of citrate synthetase. Another fraction of pyruvate is converted with involvement of pyruvate carboxylase to oxaloacetate, which is the other substrate of citrate synthetase. Thus, pyruvate produced from glycogen can assure the functioning of the citrate cycle in mitochondria and, respectively, the endogenous respiration of the cells.

But cytosolic location of pyruvate carboxylase characteristic for *S. cerevisiae* [13] imparts a specific function to the malate dehydrogenase system (which includes mitochondrial and cytosolic malate dehydrogenases, MDH1 and MDH2), which is usually considered a transport system of reducing equivalents [14]. In this case, the main function of this system seems to transport oxaloacetate into the mitochondria. Although oxaloacetate can be transported across the inner mitochondrial membrane

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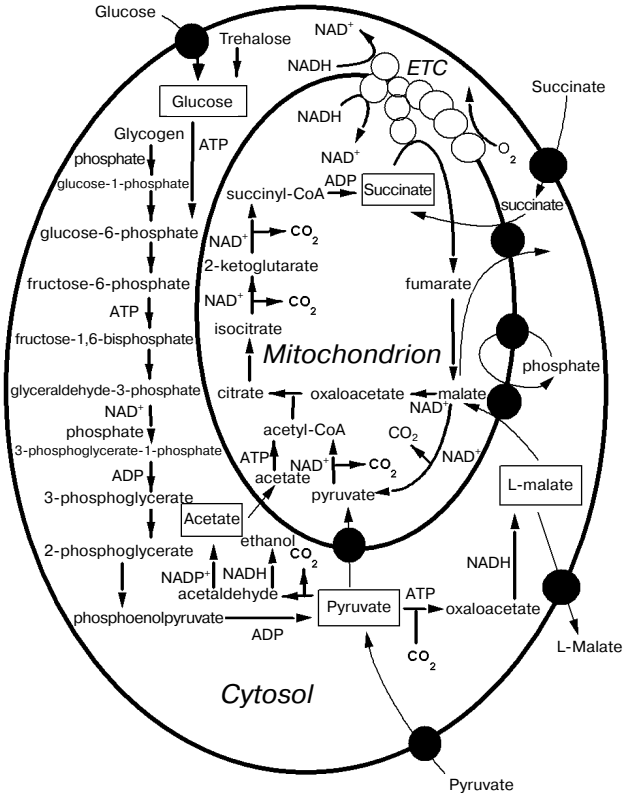

Basic metabolism and the electron transport chain (ETC) in *S. cerevisiae* cells

Scheme

by the oxaloacetate transporter, this pathway is unable to support the anapleurotic purpose of oxaloacetate, because the equimolar replacement of oxaloacetate by malate occurring in this case [15] prevents this. However, the transport of L-malate (balanced with oxaloacetate) by a dicarboxylate transporter [16] via exchange for phosphate (with involvement of an orthophosphate transporter [17]) can provide for the necessary transport of substances compensating the diminution of mitochondrial metabolites during functioning of the citrate cycle.

The present work was designed to study specific features of changes in the pool of endogenous substrates involved in the energy metabolism of *S. cerevisiae* under aerobic conditions at 0°C and the role of L-malate in this process.

MATERIALS AND METHODS

Strain and culture conditions. *Saccharomyces cerevisiae* strain Y-503 (from the collection of the State Unitary Enterprise GNII Genetika, Moscow) was used; it was obtained from the Caspian Institute of Biological Resources, Dagestan Research Center, Russian Academy of Sciences [18]. The strain was shown to belong to the *S. cerevisiae* taxon by the UP-PCR approach in the group of S. Bulat (Laboratory of Eukaryote Genetics, Department of Molecular and Radiation Biophysics, St. Petersburg Institute of Nuclear Physics, Russian Academy of Sciences). The strain Y-503 is a heterozygotic tetraploid as was shown by S. V. Benevolenskii and D. G. Kozlov (GNII Genetika).

The yeast was grown in a 750-ml flask in 100 ml of medium containing 0.2% yeast extract, 0.21% KH_2PO_4 (pH 4.5), and 0.1% glucose at 28°C with stirring on a shaker at 150 rpm. The inoculate grown in the same medium but in the presence of 1% glucose was introduced into medium preheated to 28°C, providing the initial turbidity of the suspension to be no more than 0.1 optical density unit at 540 nm. The cells cultured for 12 h were precipitated at 2700*g* for 3 min at 0°C and washed thrice in distilled water (dilution $3 \cdot 10^5$). Some experiments were performed with cells grown on synthetic medium with YNB.

Measurement of cell respiration. After removal of the culture medium, the cells were suspended in 10 mM potassium phosphate buffer (pH 5.5) at the cell concentration of 0.5 g (wet weight) per ml. The cell suspension was transferred into a flat-bottom tube, which was placed into an ice bath and intensively mixed with a magnetic stirrer. Cell respiration was measured amperometrically at 30°C in 50 mM potassium phosphate buffer (pH 5.5) in a thermostatted cell using covered electrodes [19] and recorded with a computer.

Determination of metabolites. L-Malate was determined enzymatically with "malic-enzyme" [20] on measuring NADPH concentration with a Specol 11 spectrocolorimeter (Germany) at 340 nm or an RF-5301 spectrofluorimeter (Shimadzu Scientific Instruments, Japan) at 340/460 nm (excitation/emission). To determine malate in cells, their state was fixed and low-molecular-weight compounds were extracted by a described method [21]. The fixing solution consisting of absolute ethanol and 87 mM HEPES (pH 7.5) was prepared *ex tempora*, because on storage of this solution a potassium salt of HEPES crystallizes. The determination was performed using an internal standard, with a control sample containing a definite quantity of L-malate in addition to the extract. The malate concentration in the cells was calculated on the assumption that the specific cell volume was 0.476 μl per mg dry weight [22]. Glucose in samples of the culture medium was determined with glucose oxidase with amperometric recording of oxygen [23] in 10 mM potassium phosphate buffer (pH 5.5). Previously to the determination of glucose, cells were removed by filtration though a filter with pores 0.3 μm in diameter (Synpor No. 7, Czechia).

Reagents. The following reagents were used in the study: L(–)-malic acid, oxaloacetic acid, D(+)-glucose monohydrate, HEPES, and "malic-enzyme" (Sigma, USA); yeast extract and YNB medium (Difco Laboratories, USA); sodium malonate (ICN, USA);

domestic preparations of malonic and succinic acids (recrystallized), potassium acetate (dried), and KH_2PO_4 (pure for analysis).

RESULTS

Saccharomyces cerevisiae cells were grown at low content of glucose under conditions favorable for proliferation of mitochondria [24]. The exponential growth phase (12-h culture) terminated virtually concurrently with exhaustion of exogenous glucose (Fig. 1). During this stage, the rate of endogenous respiration was high. However, the initial rate of respiration which at 30°C was, on average, 16 nmol/min per mg dry weight of the yeast was significantly lower than the respiration rate in the presence of a saturating concentration of glucose (30-60 nmol/min per mg).

Immediately after separation of cells from the culture medium, their endogenous respiration at 30°C was biphasic: the first rapid phase in 5-7 min changed for a slow phase (Fig. 2a) during which the respiration stabilized at about 15 min after the beginning of the measurement. In cells kept at 0°C under aerobic conditions the rate of endogenous respiration (v_e) decreased during the slow phase with a half-period of about 5 h (Fig. 2b). Note that the changes in the cell respiration rate depended on some specific conditions (possibly, on conditions of the medium sterilization). In Fig. 2, typical results are presented.

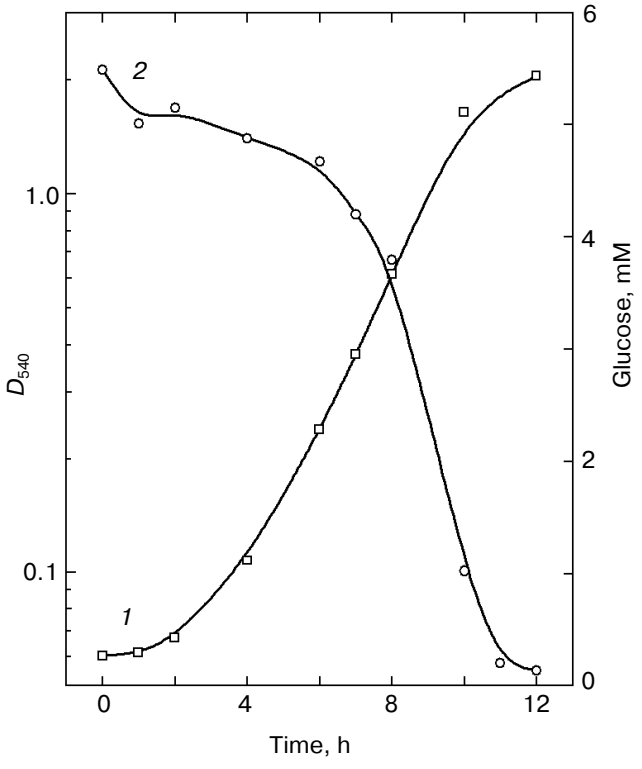

Fig. 1. Kinetics of *S. cerevisiae* strain Y-503 cell growth (*1*) and decrease in glucose content in the culture medium (*2*).

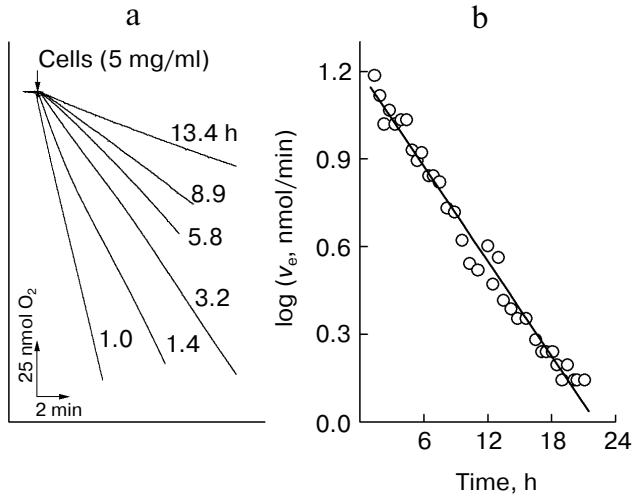

Fig. 2. Kinetics of endogenous respiration of *S. cerevisiae* cells at 30°C in 50 mM potassium phosphate buffer. a) Oxygen consumption during 1.0-13.4 h after the cell transfer from the culture medium into the incubation medium at 0°C. b) Rate dependence of the endogenous respiration (v_e) on time of cell incubation at 0°C.

In our experiments, endogenous respiration of the cells was associated only with the mitochondrial electron transport chain, as it was completely inhibited by antimycin A. The substrates of this process—pyruvate and L-malate—produced during glycogenolysis are generated in the cell cytosol [2]. Pyruvate is transported into mitochondria and oxidized via the pyruvate dehydrogenase complex. Accordingly, addition of pyruvate to freshly prepared cells stimulated cell respiration three-fivefold (data not shown). But under these conditions, exogenous L-malate poorly stimulated cell respiration. Thus, malate only 19-45% stimulated the respiration of cells grown in malate-free synthetic medium.

As it has been discussed, oxaloacetate required for functioning of the citrate cycle is produced in mitochondria from L-malate brought into them by the dicarboxylate transporter in exchange for phosphate. The oxaloacetate concentration in *S. cerevisiae* cells is very low (6-20 μM) [25, 26]; therefore, the direct measurement of its concentration in the cell compartments is associated with a difficult problem of determination of the ratio between the free and bound forms of this metabolite. Moreover, direct measurement of this metabolite cannot be sufficiently correct, because the available approaches for isolation of mitochondria [27] based on the treatment of cells with lytic enzymes at elevated temperatures prevent fixation of the metabolic state of these organelles. Therefore, in the present work we determined the level of mitochondrial oxaloacetate using an indirect approach

based on the competitive interaction between oxaloacetate and malonate in the active site of succinate dehydrogenase [28, 29].

The level of endogenous oxaloacetate in the mitochondria decreased during the incubation of cells at 0°C (Fig. 3). Thus, addition of malonate to freshly separated cells activated 1.3-1.7-fold their endogenous respiration due to displacement by malonate of oxaloacetate from the complex with succinate dehydrogenase [28, 30]. Afterwards the effect of malonate weakened (Fig. 3a), and in 5-10 h of incubation of cells at 0°C malonate ceased to activate the endogenous respiration of the cells (Fig. 3b). The addition of oxaloacetate at the concentration suppressing the cell respiration on the exogenous succinate to the level of endogenous respiration recovered the activating effect of malonate (Fig. 3b). In the experiment presented in Fig. 3, 0.4 mM oxaloacetate was required to suppress the cell respiration in the presence of succinate to the level of endogenous respiration. In this case, malonate activated the respiration by 27%. However, each of these inhibitors of succinate dehydrogenase inhibited the cell respiration in the presence of exogenous succinate (Fig. 3b). At the exogenous succinate concentration of 20 mM, 0.43 ± 0.03 mM malonate inhibited the cell respiration by 50% (two independent determinations).

It could be supposed that the decrease in the rate of endogenous respiration of the cells (Fig. 2) might be associated with a decrease in the L-malate level caused by the decrease in the content of glycogen. However, the decrease in the rate of endogenous respiration during the aerobic incubation at 0°C was accompanied by a significant increase in the level of L-malate (~threefold for 24 h) (Fig. 4). And the concentration of L-malate increased not only in the cells, but also in the culture medium when the cells were grown in both the L-malate-lacking synthetic medium and the medium with yeast extract initially containing about 12 μM L-malate. After 12 h of the cell growth, the L-malate concentration in the culture medium was increased by 21.6 ± 7.6 μM (four independent experiments).

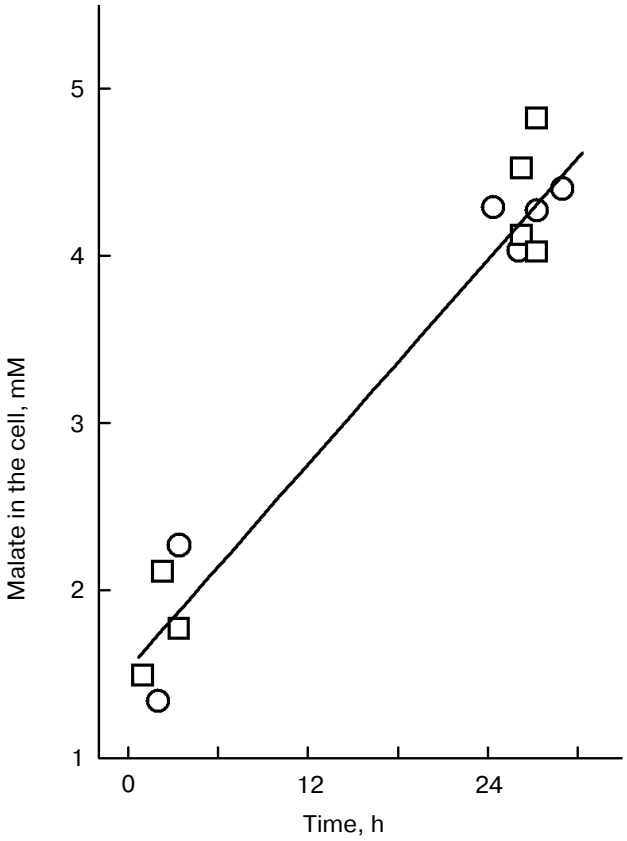

Fig. 4. Changes in the L-malate level in *S. cerevisiae* cells during aerobic incubation at 0°C in two independent experiments.

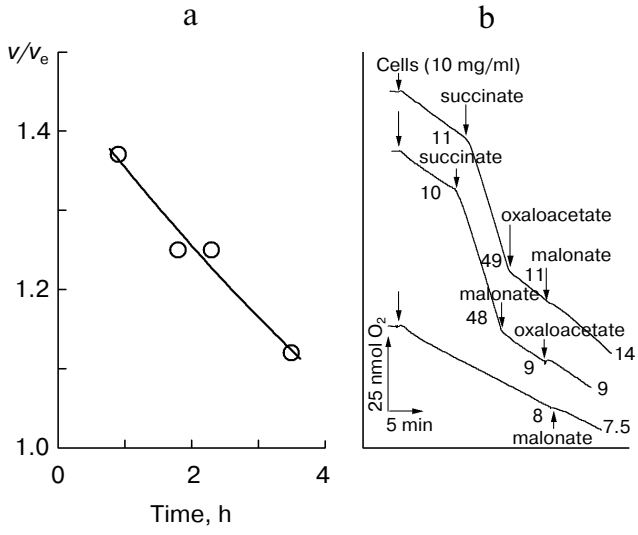

Fig. 3. Activation of *S. cerevisiae* cell respiration with malonate. a) Time-dependence of changes in the activating effect of 20 mM malonate on endogenous respiration of cells incubated at 0°C. b) Effect of 20 mM malonate and 0.4 mM oxaloacetate on the cell respiration in the presence and absence of exogenous succinate (20 mM) after incubation for 27 h at 0°C. The figures in the plot correspond to the relative rate of cell respiration.

In spite of the increase in the L-malate concentration in the cells, the respiration rate in the presence of exogenous acetate rapidly decreased (Fig. 5), as well as in the presence of glucose and pyruvate (data not presented). However, this finding was consistent with the decrease in the mitochondrial level of oxaloacetate (Fig. 3a), which is necessary for condensation with acetyl-CoA generated in this case (Scheme).

Addition of the saturating concentration of succinate (20 mM) to the freshly isolated cells only slightly stimulated their respiration. Thus, 1.4 h after the cell isolation, succinate only activated the respiration by 13%. However, with the increase in the L-malate level in the cells and decrease in the oxaloacetate concentration in the mito-

chondria *in situ*, the stimulating effect of exogenous succinate significantly increased not only absolutely (Fig. 5) but also relatively. Thus, 13.6 h after the cell isolation, the rate of their respiration was increased 3.7-fold on the addition of succinate.

Note, that the stimulating effect of succinate on the respiration was significantly different from the effect of acetate. Succinate caused a virtually full stimulation of the respiration immediately on its addition to the cells, whereas the effect of acetate (and also pyruvate and glucose) was delayed (Fig. 6). The lag-period in the case of acetate was about 5 min. Such character of the acetate effect seemed to be associated with induction of autocatalysis.

DISCUSSION

The pool of endogenous substrates of *S. cerevisiae* cells was shown to change significantly during incubation at 0°C under aerobic conditions. The exponential decrease in the respiration rate of the cells measured at 30°C (Fig. 2) after their incubation at 0°C suggests that at low temperature a process occurs with a rate fitting first order kinetics. Glycogen is the most likely substrate whose level determines the rate of the process. The level

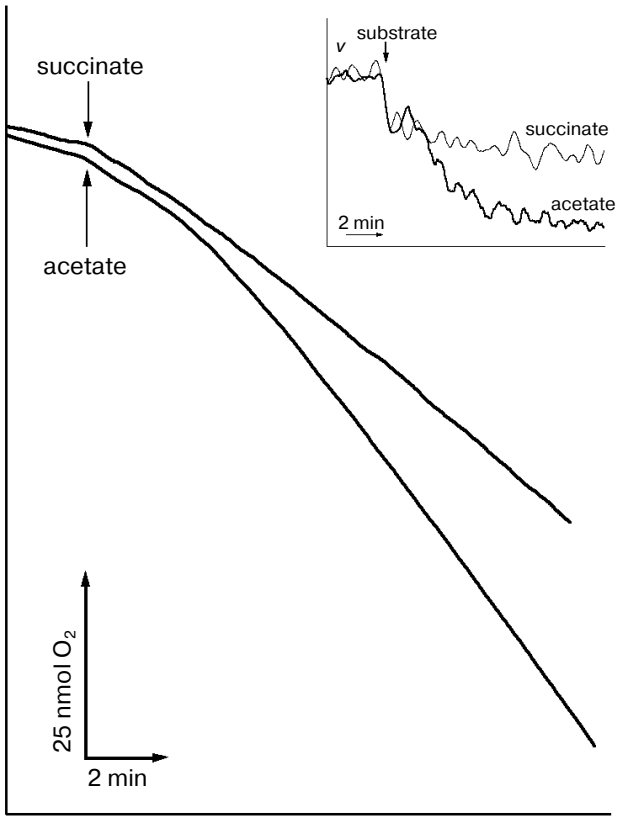

Fig. 6. Kinetics of stimulation of *S. cerevisiae* cell (5 mg/ml) respiration with succinate (10 mM) and acetate (2 mM) after 11 h of incubation at 0°C. The cells were grown on synthetic medium. (The scale of the curve of respiration with succinate is magnified twofold.) In the inset, the same data are presented in rate/time coordinates.

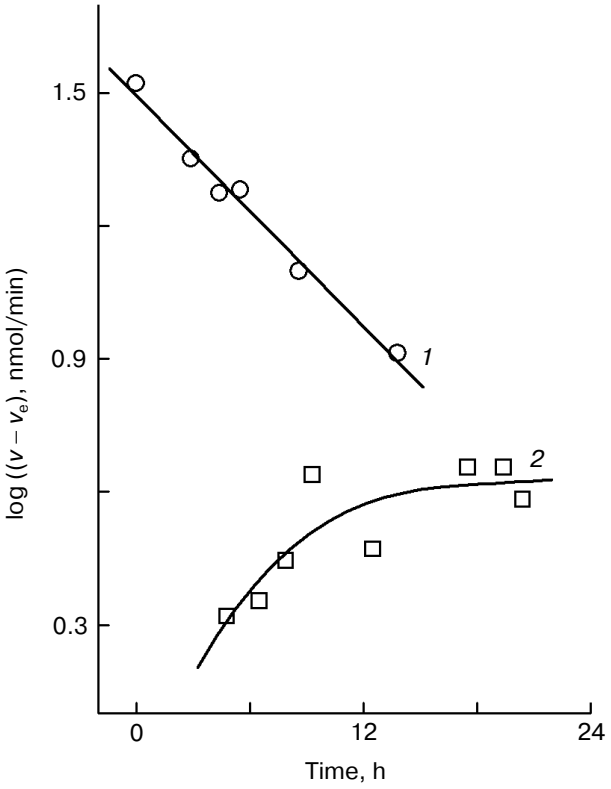

Fig. 5. Changes in the respiration rate of *S. cerevisiae* cells (5 mg/ml) incubated at 0°C with exogenous acetate (2 mM) (*1*) and succinate (20 mM) (*2*) measured at 30°C.

of this glucose polymer is known to exponentially decrease during the aerobic incubation of the cells at 30°C [1]. It seems that phosphorolysis of glycogen also occurs under "softer" conditions, i.e., at 0°C. This finding is interesting for understanding processes under natural habitat of *S. cerevisiae*, in the industrial use of the yeast, and also in experiments when the cell suspension is cooled to stabilize the metabolic state of the cells.

The decrease in the level of reserve carbohydrates would be associated with the decrease in the levels of pyruvate and L-malate due to utilization of these substrates in mitochondria (Scheme). The decrease in the level of oxaloacetate in the mitochondria would be explicable by the decrease in the level of L-malate (Fig. 3). But the concentration of L-malate in the cell not only failed to decrease, but on the contrary, increased significantly (Fig. 4). This suggests that the decrease in the production of malate is associated with the more rapid inhibition of its utilization.

The decrease in the rate of cell respiration along with the concurrent increase in the malate level in the cells might be explained by the increased level of malate in

mitochondria leading to the increase in the level of oxaloacetate and, as a consequence, inhibition of succinate dehydrogenase and cell respiration. In freshly isolated cells, the high rate of the acetyl-CoA production from pyruvate should be accompanied by a high rate of oxaloacetate production. Despite a relatively high level of oxaloacetate, under these conditions the high rate of succinate production is maintained, partially preventing a stable complexing of succinate dehydrogenase with oxaloacetate. The activating effect of malonate on the cell respiration during the initial stages of incubation at 0°C (Fig. 3) suggests that oxaloacetate can control the rate of the citrate cycle and thus slow down the rate of L-malate oxidation in mitochondria of intact cells. This can increase the malate level in the cell.

However, during the cell incubation at 0°C the fraction of succinate dehydrogenase free of oxaloacetate increases (Fig. 3). This could occur due to decrease in the level of oxaloacetate because of its accelerated utilization, resulting in the decrease in the malate level in the cytosol. However, on retention of the interrelationship between the cytosolic and mitochondrial pools of malate, this contradicts the observed increase in the malate level in the cell (Fig. 4).

Thus, to explain our findings, it remains to suppose that at low temperature the anapleurotic function of malate (oxaloacetate) provided for by the malate transport via the mitochondrial dicarboxylate transporter should be disturbed (Scheme). This disturbance seems to be caused by the significantly higher activation energy of the transporter-catalyzed transport than the enzyme-catalyzed reactions [31]. Therefore, on the temperature decrease from 30 to 0°C, the rate of metabolite transport begins to determine the rate of their utilization, and just this results in the decrease in the oxaloacetate and malate levels in the mitochondria and the increase in their contents in the cytosol.

The poor stimulating effect of exogenous malate and succinate on the respiration of freshly isolated cells seems to be associated with high initial concentrations of the corresponding endogenous substrates in the mitochondria. The acceleration of oxidation of the exogenous succinate with increase in the malate level in the cell (Fig. 5) can be due to acquisition by malate, on the temperature increase from 0 to 30°C, of the ability not only to leave the cells but also enter the mitochondria in exchange for phosphate (Scheme). The increase in the malate concentration in the mitochondria, in its turn, activates the Chappell cycle [32], according to which the level of succinate, which is a source for L-malate generation, increases due to exchange for this dicarboxylate. This is promoted by the significantly higher affinity of the mitochondrial dicarboxylate transporter for L-malate than phosphate [33].

As mentioned, malate is produced in the cytosol due to pyruvate carboxylation. Because mitochondria are the main source of carbon dioxide (Scheme), the L-malate concentration in the cell can be increased at the cost of the mitochondrial pool of the substrates. The renewal of the mitochondrial transport of malate with increase in the temperature seems to be insufficient to recover this pool. This is shown by the observed decrease in the rate of oxidation of exogenous acetate (Fig. 5) and also glucose and pyruvate. These substrates could provide for the utilization of oxaloacetate needed for the oxidation of malate. But to activate this pathway, an efficient generation of the transmembrane potential in mitochondria is necessary, because the transport of pyruvate catalyzed by the monocarboxylate transporter (accompanying cell respiration on glucose or pyruvate) is co-imported with a proton [34, 35]. Acetate penetrates into the mitochondria by diffusion as acetic acid, i.e., its transport is also associated with the entrance of protons into the mitochondrial matrix. Thus, for oxidation of glucose, pyruvate, and acetate protons must be removed from the mitochondria. Moreover, the utilization of acetate with involvement of acetyl-CoA synthetase requires synthesis of ATP. The removal of protons and ATP synthesis in mitochondria occur due to generation of the transmembrane potential. In the early stages of exhaustion of the pool of mitochondrial substrates acetate seems to promote the partial recovery of this pool. This seems to be the explanation of a gradual development of respiration in the presence of acetate (Fig. 6).

Thus, our findings can be explained by the known properties of the enzymes and transporters functioning in *S. cerevisiae* cells. But it should be noted that the proposed interpretation is not the only possible one, and it needs be tested experimentally. In particular, this concerns the role of the decarboxylating malate dehydrogenase located in the mitochondria of *S. cerevisiae* [36], which probably recovers the mitochondrial pool of the substrates. But the unique kinetic parameters of this enzyme (its K_m for malate is ~50 mM [37]) seem to exclude such a possibility. The role of the NAD redox-state in the control of the malate/oxaloacetate ratio in the cell compartments is also not discussed. The balance between the NAD^+/NADH ratio in the cytosol and mitochondrial matrix can be maintained due to the presence in the mitochondria of two NADH dehydrogenases, external and internal (Scheme). However, at present this process is not sufficiently understood [38].

The hypothesis about the removal of excess malate via simple diffusion of this dicarboxylate across the plasma membrane seems unlikely, because at pH 7 of the cytosol [39] malate is a polar dianion with low permeability across the cell membrane [40]. Therefore, the metabolism is likely to be corrected by a dicarboxylate transporter of the plasma membrane similar to the transporter found in the yeast *Kluyveromyces lactis* [41].

The plasma membrane of *S. cerevisiae* is believed to lack a system of dicarboxylate transport [41-43]. The

weak stimulation by malate and succinate of the freshly isolated cells in our experiments (see above) seems to favor this viewpoint. But the results obtained in the further stages of cell incubation at 0°C, when succinate considerably stimulates the malonate-sensitive respiration, contradicts this idea (Figs. 3 and 5). This suggests that the plasma membrane of this yeast has to contain a dicarboxylate transporter. It seems that the physiological function of this transporter in *S. cerevisiae* could be not the utilization of dicarboxylates, but removal of excess malate from the cell. This viewpoint correlates with the data on L-malate secretion during the cell growth (see above). In this connection, studies on the mechanism of dicarboxylate transport across the plasma membrane of *S. cerevisiae* seem to be especially interesting.

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