Microbiology, Vol. 74, No. 5, 2005, pp. 527–532. Translated from Mikrobiologiya, Vol. 74, No. 5, 2005, pp. 609–615.

EXPERIMENTAL ARTICLES

The Effect of Succinate on Respiration, Transamination, and Pyruvate Formation in Cells of the Yeast *Dipodascus magnusii*

A. P. Il'chenko*,[1], D. I. Ogorelyshev, N. V. Shyshkanova*, A. P. Sokolov*, T. V. Finogenova*, and M. N. Kondrashova****

** Skryabin Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, pr. Nauki 5, Pushchino, Moscow oblast, 142290 Russia*

*** Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow oblast, 142290 Russia*

Received November 18, 2004; in final form, May 12, 2005

Abstract—The effect of succinate on the growth and respiration of the yeast *Dipodascus magnusii* VKM Y-1072, which is auxotrophic for thiamine and biotin, was studied. The addition of succinate to a culture grown on glucose was found to activate the respiration of cells on various substrates by enhancing the processes related to transamination reactions. In this case, aerobic fermentation (ethanol production) decreased, whereas pyruvate production increased. When succinate was added to the medium as the sole carbon source, it supported yeast growth in the absence of one of the two vitamins, thiamine or biotin, but not both. The yeast metabolism was completely respiratory, without any signs of aerobic fermentation. A drastic rise in pyruvate production in the yeast grown on glucose in the presence of succinate and the absence of biotin are also indicative of metabolic changes.

Key words: succinate, pyruvate, transamination, the Krebs cycle.

The adrenaline-induced activation of energy metabolism and physiological functions in animals changes oxidative processes toward a pathway that is characterized by a higher degree of succinate oxidation than in the case of the complete Krebs cycle (the tricarboxylic acid cycle, the TCA cycle) [1–3]. As a result, oxidative phosphorylation, by means of shunting the Krebs cycle through the transamination of glutamate with oxaloacetate, is accelerated. This process enhances the formation of succinate via α-oxoglutarate or directly from glutamate. Similar events were described by Kondrashova for animal cells in response to low doses of succinate, which stimulates the secretion of adrenaline [3], and by Coleman for actively proliferating cells (the so-called truncated Krebs cycle) [4].

The enhanced oxidation of succinate in mammalian cells stimulates pyruvate formation [1, 2, 5], which produces a curative effect on metabolic acidosis, since low doses of succinate considerably elevate the level of pyruvate and diminish the level of lactate [3]. The increased formation of pyruvate correlates with the activation of alanine and aspartate transaminases and phosphoenolpyruvate formation but not with the activation of lactate dehydrogenase [3]. This circumstance indicates that there is an inflow of mitochondrial pyruvate, which is produced in the transamination reactions occurring in the truncated oxidative cycle. The improved contribution of succinate to oxidation enhances mitochondrial phosphorylation, suppresses glycolysis, and reduces lactate formation [3]. In other words, succinate intensifies the Pasteur effect.

In contrast to higher eukaryotes, the final product of fermentation in yeasts is ethanol not lactate. They are both produced from the same final product of glycolysis, i.e., pyruvate. The formation of ethanol and lactate is activated under hypoxic conditions. Oxygen inhibits ethanol fermentation (the Pasteur effect). However, some aerobically grown yeasts can actively ferment glucose [6–9]. This aerobic fermentation is analogous to the Crabtree effect typical of malignant tissues. Also of considerable interest are the aforementioned phenomena of glycolysis suppression and activation of oxidative processes by succinate.

It should be noted that the effect of various doses of succinate on glucose metabolism has been sufficiently studied in animals but not in yeasts. The aim of this work was to study whether succinate exerts similar effects on the glucose metabolism of lower unicellular organisms (yeasts) and higher eukaryotes.

[1] Corresponding author; e-mail: ilchenko@ibpm.pushchino.ru

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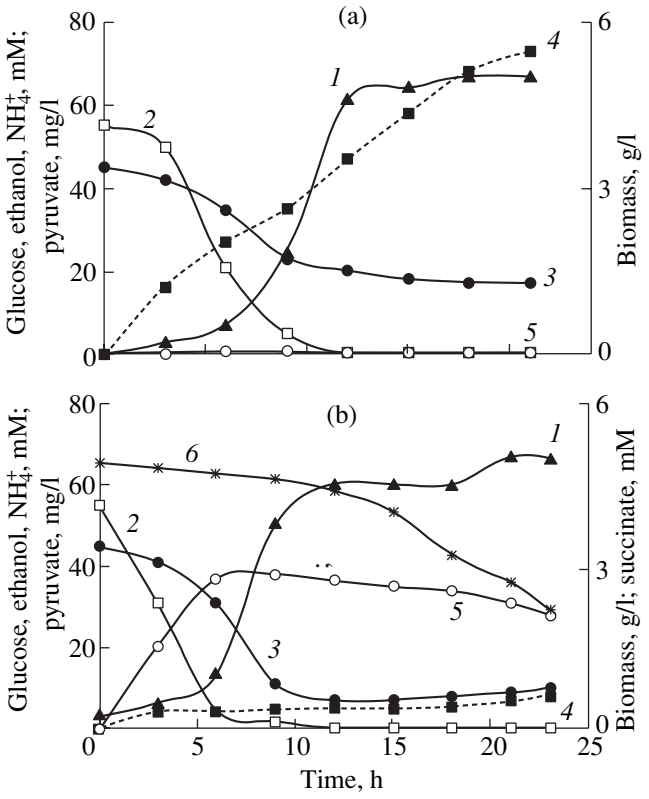

Fig. 1. Batch cultivation of *D. magnusii* on 1% (55.4 mM) glucose in fermentor (a) without and (b) in the presence of 5 mM succinate: (*1*) biomass; (*2*) glucose; (*3*) ammonium; (*4*) ethanol; (*5*) pyruvate; and (*6*) succinate.

MATERIALS AND METHODS

The experiments were carried out with the yeast *Dipodascus magnusii* VKM Y-1072 [10]. This yeast is auxotrophic for thiamine and biotin, which implies that it is unable to grow on glucose and sucrose in the absence of these vitamins.

The yeast was cultivated either in 750-ml flasks containing 100 ml of mineral Reader medium (pH 5.5) or in a 3-l fermentor containing 1.5 l of the same medium. The complete medium was supplemented with 10 μg/l biotin and 250 μg/l thiamine. The concentration of oxygen in the fermentor was maintained at a level of p_{O_2} = 20% oxygen saturation. The carbon sources were glucose and succinate. The concentrations of glucose and NH_4^+ were maintained at levels of 27.7 ± 5 and 20 ± 5 mM, respectively, by fractionated feeding of the substrates. In these experiments, the concentration of glucose was determined polarographically with glucose oxidase and that of NH_4 was measured with an ion-selective electrode (Orion). The measurements were carried at 3-h intervals.

Alternatively, the concentration of glucose was measured photometrically at 480–520 nm using a reagent kit from ZAO Diakon-DS (Russia).

Ethanol was determined polarographically by measuring the consumption of oxygen in the presence of methanol oxidase isolated from the yeast *Hansenula polymorpha* [11].

In order to measure the concentration of succinate and pyruvate in the culture liquid, the yeast cells were removed by centrifugation at 14000 *g* for 5 min. The supernatant was deproteinized by adding an equal volume of 6% perchloric acid and removing the precipitate by centrifugation at 14000 *g* for 15 min. The final supernatant was used for analysis.

Organic acids were analyzed by HPLC using an Aminex HPX-87H column (300 × 7.8 mm) from Bio-Rad. The mobile phase was 4 mM H_2SO_4. The column was kept at 35°C. The elution rate was 0.6 ml/min. The eluted compounds were detected at 210 nm. The calibration curve was constructed using a standard kit from Bio-Rad.

The respiration rate of the intact yeast cells was measured at 30°C using a Clark-type oxygen electrode. The respiration medium contained 20 mM KH_2PO_4 and 100 mM NH_4HCO_3 (pH 5.5). If the intact cells were unable to oxidize particular substrates, they were permeabilized with digitonin. For this purpose, 10-h-old cells grown on glucose + 5 mM succinate were incubated at 30°C in 50 mM KH_2PO_4 (pH 5.5). Then, the cell suspension, containing 0.8–1.5 mg dry wt cells/ml, was supplemented with digitonin to a final concentration of 0.2–0.3 mg/ml. The period of incubation with digitonin was chosen experimentally. The incubated cells were precipitated by centrifugation at 7000 *g*, washed twice with an incubation medium containing no digitonin, and resuspended in the last medium.

RESULTS

The effect of succinate on the respiration and growth of *D. magnusii* cells on glucose. Figure 1a shows the results of batch cultivation of the yeast in the complete medium in the fermentor at the initial glucose concentration, equal to 1% (55.4 mM). It is evident from this figure that yeast growth was accompanied by the accumulation of ethanol in the medium. In other words, in spite of intense aeration, the yeast cells did accomplish aerobic fermentation. In this case, the oxidation rates of ethanol and glucose were sufficiently high (see table).

Since intact cells were found to be able to oxidize glucose, ethanol, and pyruvate but not the Krebs cycle intermediates, the oxidation of these intermediates was studied using permeabilized cells (table). As is evident from the table, the highest oxidation rates were observed for ethanol and exogenously added NADH.

The addition of 5 mM succinate to the medium when it contained only glucose drastically diminished the production of ethanol and augmented the excretion of pyruvate (Fig. 1b). Consumption of the nitrogen source was 2.5 times higher in the presence of succinate

Oxidation rates of various substrates (nmol O_2/(min mg dry wt) by *D. magnusii* cells

Growth substrate	Vitamins added	Oxidized substrates			
		Glucose	Ethanol	NADH	Succinate
1% Glucose***	Thiamine, biotin	100 ± 15	180 ± 10	162 ± 8*	38 ± 11*
1% Glucose + 5 mM succinate***	Thiamine, biotin	167 ± 23	422 ± 12	784 ± 14*	395 ± 8*
1.2% Succinate***	Thiamine, biotin	215 ± 12	584 ± 15	867 ± 7	345 ± 12
1.2% Succinate***	Thiamine	138 ± 15	367 ± 22	–	519 ± 9
1.2% Succinate***	Biotin	154 ± 17	786 ± 10	–	213 ± 10
1% Glucose + 5 mM succinate**	Thiamine	354 ± 10	2180 ± 10	2042 ± 6*	356 ± 16*

Growth substrate	Oxidized substrates					
	α-Oxoglutarate	Pyruvate	Oxaloacetate	α-Oxoglutarate + aspartate	Glutamate + oxaloacetate	Pyruvate + glutamate
1% Glucose***	51 ± 4*	42 ± 3	34 ± 3	112 ± 5*	152 ± 9*	75 ± 5*
1% Glucose + 5 mM succinate***	102 ± 3*	118 ± 7	73 ± 5	274 ± 11*	311 ± 15*	200 ± 9*
1.2% Succinate***	165 ± 7	97 ± 4	187 ± 7	364 ± 10	567 ± 25	215 ± 7
1.2% Succinate***	386 ± 18	131 ± 4	112 ± 10	386 ± 17	474 ± 17	486 ± 16
1.2% Succinate***	31 ± 3	211 ± 9	231 ± 5	184 ± 25	512 ± 8	697 ± 20
1% Glucose + 5 mM succinate**	378 ± 15*	216 ± 8	223 ± 8	489 ± 16*	368 ± 11*	369 ± 11*

* Permeabilized cells.
** Constant concentrations of glucose and nitrogen source.
*** Batch cultivation.

than in its absence, although the biomass accumulated in these two cases was approximately the same. These data suggest that yeast cultivation on glucose in the presence of succinate suppresses aerobic fermentation and stimulates nitrogen metabolism.

The intact yeast cells grown on glucose in the presence of succinate showed higher oxidation rates (by 2–3 times) of ethanol and glucose as compared to the cells grown on glucose without succinate (table). The oxidation of the other substrates by the former cells was very slow. For this reason, further experiments with these cells were carried out after their permeabilization with digitonin (table).

The oxidation of all of the substrates by these permeabilized cells was 2–3 times more rapid than by the cells grown on 1% glucose alone. The oxidation of α-oxoglutarate, oxaloacetate, and pyruvate by the cells grown on glucose in the presence of succinate considerably increased in the presence of amino acids and was inhibited (by 30–40%) by aminohydroxyacetate (the specific inhibitor of transamination reactions). The control experiments showed that the amino acids themselves were oxidized at a low rate.

Thus, exogenously added succinate substantially intensifies cell respiration and suppresses aerobic fermentation (but not glycolysis); that is, it intensifies the Pasteur effect. The enhanced consumption of NH_4^+ (Fig. 1b) and the rapid oxidation of the substrate pairs α-oxoglutarate + aspartate, oxaloacetate + glutamate, and pyruvate + glutamate by the cells grown on glucose in the presence of succinate suggest the activation of transamination reactions.

The growth and respiration of *D. magnusii* cells on succinate. As was mentioned in Materials and Methods, the yeast *D. magnusii* is auxotrophic for thiamine and biotin; in other words, it is unable to grow in the absence of these vitamins.

In spite of the fact that the yeast did not grow on 1% glucose under high aeration in the absence of one of the two vitamins, the consumption rate of glucose was high and the yeast cells produced ethanol (data not shown). The addition of the initially absent vitamin restored the intense growth of the culture and intensified cell respiration.

It should be noted that the fermentative metabolic shift of the yeast *Endomyces magnusii* when grown under aerobic conditions on fermentable sugars (such as sucrose) in the absence of one vitamin (biotin) or an

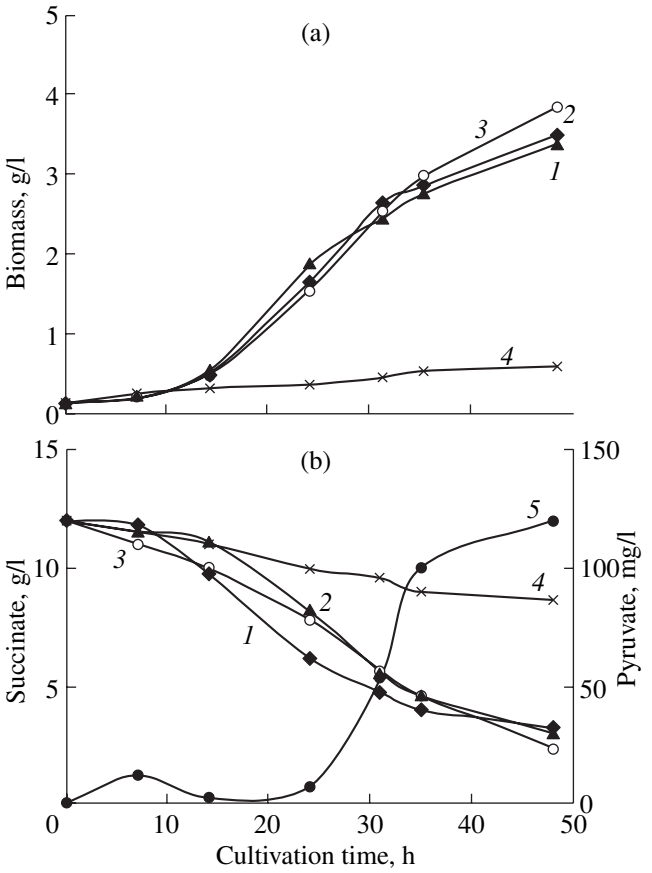

Fig. 2. (a) Growth of *D. magnusii* on 1.2% (101 mM) succinate and (b) its consumption in the presence of (*1*) thiamine and biotin, (*2*) biotin alone, (*3*) thiamine alone, and (*4*) in the absence of both vitamins. Curve *5* shows pyruvate excretion during growth in the absence of both vitamins (no appreciable pyruvate excretion was noted in other cases).

excess of the other (thiamine) has long been known about [12, 13].

Figure 2a shows the accumulation of biomass during the cultivation of *D. magnusii* on 1.2% succinate in the presence of one of the vitamins. In the control experiments, the yeast was grown either in the presence or in the absence of both vitamins. The 1.2% (101 mM) concentration of succinate was chosen so as to equalize the carbon content of 1% glucose (55.5 mM). As can be seen from Fig. 2a, the absence of one vitamin (either biotin or thiamine) did not notably affect yeast growth, whereas the absence of both vitamins completely arrested the growth. Similar data were obtained for the succinate consumption (Fig. 2b). Growth on succinate was not accompanied by the accumulation of ethanol in the medium.

Thus, unlike glucose, succinate is probably able to support yeast growth in the absence of either biotin or thiamine due to compensation for a deficiency of thiamine-dependent enzymes (in the absence of thiamine) or biotin-dependent enzymes (in the absence of biotin).

The experimental data on respiration of the succinate-grown cells confirmed the existence of metabolic changes dependent on thiamine and biotin deficiency (Fig. 3, table). Unlike the glucose-grown cells, the succinate-grown cells showed weak endogenous respiration and were able to oxidize various substrates both individually and in combination. For this reason, the succinate-grown cells were not subjected to permeabilization.

Yeast cells grown in the presence of biotin but without thiamine readily oxidize pyruvate. It is known that thiamine deficiency affects the dehydrogenase activity but not the decarboxylase activity of the pyruvate dehydrogenase complex [14]. The oxidation of pyruvate was considerably enhanced by aspartate and glutamate and suppressed by aminohydroxyacetate, indicating the functioning of aspartate aminotransferase and alanine aminotransferase. Clearly, the succinate-grown cells readily oxidized succinate. At the same time, they only slightly oxidized α-oxoglutarate, isocitrate, and citrate (intermediates of the right branch of the TCA cycle).

In contrast, the yeast cells grown in the presence of thiamine but without biotin readily oxidized both α-oxoglutarate and pyruvate (Fig. 3, table). The oxidation of α-oxoglutarate was enhanced by aspartate and suppressed by aminohydroxyacetate (by 35–40%). The subsequent addition of malonate, the specific inhibitor of succinate dehydrogenase, elevated the degree of inhibition. The substrate pairs oxaloacetate + glutamate and pyruvate + glutamate were oxidized at a high rate.

Thus, yeast growth on succinate alone or on glucose + succinate suppresses aerobic fermentation and stimulates transamination reactions.

Succinate consumption and pyruvate formation. As was mentioned above, *D. magnusii* cells are unable to grow on glucose when either biotin or thiamine is absent, but they can utilize succinate as a carbon source and grow on it in the absence of one of the two vitamins. This circumstance suggests that succinate metabolism allows the dependence of yeast growth on thiamine- or biotin-dependent enzymes to be avoided.

Moreover, our experiments showed that the yeast can grow on glucose + succinate in the absence of biotin (Fig. 4). These experiments were carried out at a constant concentration of glucose and NH_4^+, as described in Materials and Methods. The addition of 5 mM succinate to the glucose-containing cultivation medium intensified culture growth and led to complete consumption of the ethanol from the medium. The yeast cells grown under these conditions oxidized the substrate pairs α-oxoglutarate + aspartate, oxaloacetate + glutamate, and pyruvate + glutamate more readily and showed a higher degree of inhibition of this oxidation by aminohydroxyacetate as compared to the cells grown on glucose alone (table).

Further additions of succinate did not influence the growth dynamics but did suppress the accumulation of ethanol in the medium (Fig. 4). In contrast, the excre-

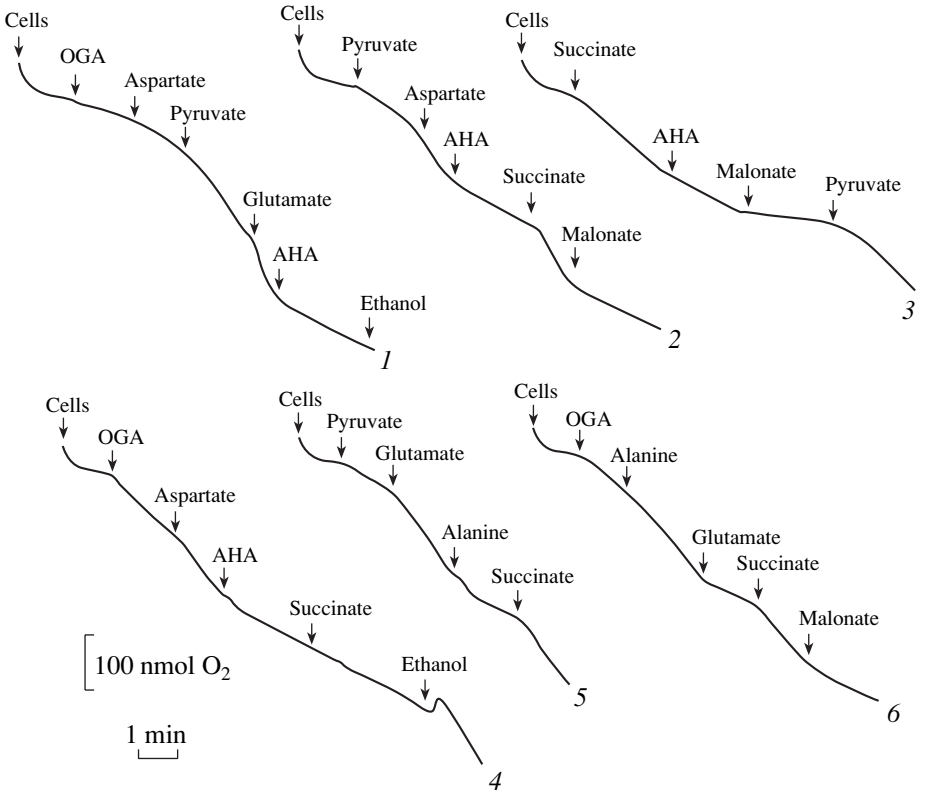

Fig. 3. Oxidation of various substrates by *D. magnusii* cells grown on 1.2% (101 mM) succinate in the absence of thiamine (curves *1–3*) and biotin (curves *4–6*). Additions: cells, 0.85 mg dry wt; glutamate, 3 mM; aspartate, 6 mM; alanine, 5 mM; pyruvate, 5 mM; α-oxoglutaric acid (OGA), 5 mM; succinate, 3 mM; ethanol, 100 mM; aminohydroxyacetate (AHA), 4 mM; and malonate, 3 mM.

tion of pyruvate drastically increased; consequently, its concentration in the medium (2.2 g/l) was an order of magnitude higher than during growth on glucose in the presence of both vitamins (Fig. 1a). These data suggest that the first addition of succinate stimulates the respiration of yeast cells and their growth on glucose in the absence of biotin, whereas further additions of succinate stimulate the excretion of pyruvate.

DISCUSSION

The addition of 5 mM succinate to the *D. magnusii* culture grown on glucose activated the oxidation of various substrates (table) and stimulated transamination reactions, as is evident from the more intense oxidation (as compared to the cells grown on glucose alone) of the substrate pairs α-oxoglutarate + aspartate, oxaloacetate + glutamate, and pyruvate + glutamate and its inhibition by aminohydroxyacetate. This suggestion is confirmed by the faster consumption of NH_4^+ under these conditions (Fig. 1). Changes in the aerobic metabolism of glucose in response to the addition of 5 mM succinate also manifest themselves in a decrease in the amount of ethanol accumulated in the medium; that is, in the suppression of aerobic fermentation.

Distinct metabolic changes also occur when the yeast grows on succinate alone. The *D. magnusii* cells can grow well in the absence of either thiamine or biotin when succinate is used as the carbon source (Fig. 2). This circumstance suggests that succinate metabolism allows the dependence of yeast growth on thiamine- or biotin-dependent enzymes to be avoided. Moreover, the yeast can grow on glucose + succinate in

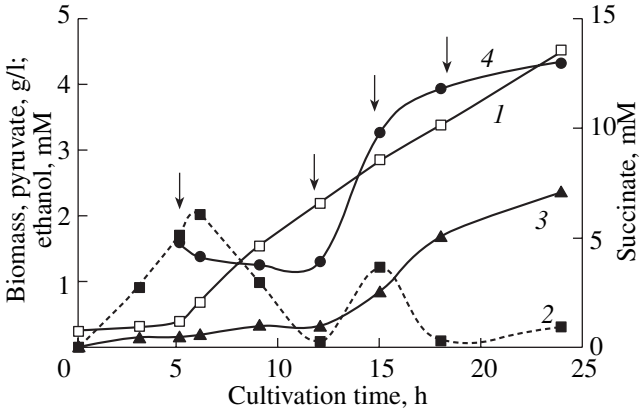

Fig. 4. Effect of succinate on the growth of *D. magnusii* in the absence of biotin at constant concentrations of glucose (27.7 ± 5 mM) and NH_4^+ (20 ± 5 mM). Curves: (*1*) biomass; (*2*) ethanol; (*3*) pyruvate; (*4*) succinate. The arrows indicate the times of succinate additions.

the absence of biotin (Fig. 4). These data, together with those showing an enhancement of oxidative and transamination reactions (Fig. 3, table), suggest that the yeast can grow in the absence of one of the two vitamins, which are coenzymes of pyruvate decarboxylation and carboxylation, due to the functioning of alternative pathways of pyruvate metabolism.

Pyruvate is not only formed in glycolysis. Moreover, during yeast growth on succinate, hardly any of the pyruvate is glycolytic. It is formed either in the truncated TCA cycle [1–4] or via transamination and decarboxylation of malate (or oxaloacetate).

When the yeast grows on the complete medium with glucose, a small amount of pyruvate is excreted. The cultivation of *D. magnusii* on glucose in the presence of 5 mM succinate substantially intensifies pyruvate excretion (Fig. 2). The absence of biotin in the medium enhances pyruvate excretion still further (Fig. 4).

The data presented in Fig. 4 indicate that the first addition of succinate stimulates the growth of *D. magnusii* on glucose in the absence of biotin, although the concentration of added succinate changes insignificantly. Further additions of succinate stimulate both yeast growth (Fig. 4) and excretion of pyruvate (up to 2 g/l).

The concentration of the succinate taken up by yeast cells (but probably not utilized) throughout the cultivation period did not exceed 5 mM (~0.5 g/l), whereas the biomass rose from 0.25 to 4.5 g/l following the first succinate addition. This result can be explained in terms of the diauxie phenomenon, when an easily utilizable carbon source (primarily glucose) suppresses consumption of all the other added substrates or excreted products as long as the concentration of the first substrate falls to zero [8]. As is evident from the data presented in Fig. 1b, succinate (5 mM) consumption began only after the culture had entered the stationary phase for glucose.

To conclude, the pyruvatogenic effect of exogenously added succinate in the yeast *D. magnusii* is the result of two processes: (1) trigger activation of oxidative and transamination reactions at low concentrations of succinate (5 mM) and (2) enhanced excretion of pyruvate into the medium at higher succinate concentrations. The first process resembles that observed in animals, when secreted adrenaline stimulates the truncated TCA cycle [3].

ACKNOWLEDGMENTS

This work was supported by the President of the Russian Federation under the program Leading Scientific Schools, grant no. NSh-824.2003.4.

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