ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 4, pp. 450–455. © Pleiades Publishing, Ltd., 2010.
Original Russian Text © A.P. Ilchenko, V.Ya. Lysyanskaya, T.V. Finogenova, I.G. Morgunov, 2010, published in Mikrobiologiya, 2010, Vol. 79, No. 4, pp. 475–480.

EXPERIMENTAL ARTICLES

Characteristic Properties of Metabolism of the Yeast *Yarrowia lipolytica* during the Synthesis of α-Ketoglutaric Acid from Ethanol

A. P. Ilchenko†, V. Ya. Lysyanskaya, T. V. Finogenova[1], and I. G. Morgunov

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

Received October 1, 2009

Abstract—The study of free amino acid content in *Yarrowia lipolytica* cells grown on ethanol under thiamine deficiency showed that glutamate, alanine, and γ-aminobutyric acid (γ-ABA) occurred in the highest concentrations among the present 17 free amino acids. The culture liquid contained no amino acids. Analysis of the enzymes of oxidative metabolism in the yeast grown under these conditions showed that the cell-free homogenate contained substantial activity of glutamate decarboxylase, γ-ABA transaminase, and succinyl semialdehyde dehydrogenase. This result indicated the formation of succinate from glutamate in a reaction catalyzed by 4-aminobutyrate aminotransferase (γ-aminobutyrate bypass) under severe thiamine deficiency. These studies lead to the conclusion that cultivation of the yeast *Y. lipolytica* on ethanol under thiamine deficiency causes adaptive stress-induced metabolic changes. Increase of ammonium nitrogen consumption and excretion of α-ketoglutaric acid are indicative of physiological changes, the functioning of the γ-aminobutyrate bypass and high activity of malate dehydrogenase are manifestations of metabolic changes, and increased activities of the transamination reactions reflect the changes in nitrogen metabolism.

Key words: yeasts, free amino acids of a cell, TCA cycle, γ-aminobutyric acid, α-ketoglutaric acid, succinate, transaminases, succinyl semialdehyde.

DOI: 10.1134/S0026261710040065

On the basis of results obtained in our previous work [1], we have suggested that succinate is produced in the cells of the yeast *Yarrowia lipolytica* during their cultivation under thiamine deficiency via glutamate decarboxylation with the formation of γ-aminobutyric acid.

4-Aminobutyric acid (4-ABA or γ-ABA) is widely represented in both prokaryotic and eukaryotic cells. This amino acid, which is not a constituent of proteins, makes up a considerable part of the pool of free amino acids in eukaryotes, including yeasts [2–7]. It has been established that γ-ABA is a product of glutamate metabolism (Fig. 1) during its decarboxylation by glutamate decarboxylase (GDC).

The balance between γ-ABA synthesis and degradation is regulated by the ratio of the activities of GDC, γ-ABA transaminase, and succinyl semialdehyde dehydrogenase [8]. The final product of γ-ABA degradation in mammals is succinate. At the same time, γ-ABA degradation in plants and microorganisms under certain conditions may result in formation of 4-hydroxybutyrate (γ-hydroxybutyrate) and not succinate [9].

As is known, γ-ABA and glycine are the main inhibiting mediators of the brain and spinal cord (in contrast to glutamate and aspartate, which are exciting mediators). Impaired biosynthesis and secretion of γ-ABA in humans leads to various diseases, including epilepsy and Parkinson's disease [10, 11].

In plants and microorganisms, the function of γ-ABA has been studied much less. It has been established that they can utilize this amino acid as a carbon and nitrogen source [12, 13]. γ-ABA rapidly accumulates in yeast cells in response to different abiotic stresses, such as hypoxia, cooling, or oxidative stress [6]. For example, the presence of the γ-ABA-forming enzyme, glutamate decarboxylase, determines the high resistance of *S. cerevisiae* cells to oxidative stress [6].

Cultivation of yeasts under deficiency of nitrogen, vitamins, or trace elements, resulting in both physiological and metabolic changes, may also be termed a stress situation. For example, cultivation of the yeast *Y. lipolytica* on ethanol under thiamine deficiency [1] causes overproduction and excretion of α-ketoglutaric acid (KGA). Hence, the goal of our work was to study adaptive metabolic changes under cultivation of the yeast *Yarrowia lipolytica* under conditions of thiamine deficiency.

† Deceased.

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

MATERIALS AND METHODS

Object of research. The mutant strain of yeast *Yarrowia lipolytica* no. 1 was selected as an active producer of α-ketoglutaric acid from ethanol [14]. The strain is maintained on a mineral–salt medium with paraffin at the Laboratory of Aerobic Metabolism of Microorganisms, Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences.

Cultivation conditions. The yeast was grown in an ANKUM-2M fermentor, working volume 1.5 l, under conditions described previously [15, 16]. Ethanol (1–2.5 g/l) was used as a carbon source. The thiamine concentration under conditions of KGA synthesis was 3 μg/l. Depending on the objectives of the experiments, $(NH_4)_2SO_4$ was added as a nitrogen source once or continuously in proportion to its consumption to maintain the (NH_4^+) concentration at a level of 1.4–1.6 g/l.

Detection of organic acids and amino acids. The content of organic acids in the cultivation medium was determined by isocratic HPLC on an Aminex HPX-87H column, 300 ± 7.8 mm (Bio-Rad), as described previously [17].

For detection of free amino acids in the yeast cells, the biomass was lyophilized. Then, 2 ml of 80% HCl-acidified ethanol was added to 20–30 mg of dry biomass and incubated for 24 h at room temperature. The resultant extract was centrifuged, the pellet was removed, and the supernatant was studied in an amino acid analyzer by the method of Spackman et al. [18]. The results of analysis were calculated by the method of absolute calibration.

Free amino acids in the culture liquid were detected after treatment with 3% trichloroacetic acid by the same method [18].

Determination of enzyme activities. The methods of cell disintegration, obtaining of cell-free homogenate, and determination of activity of the enzymes of the tricarboxylic acid (TCA) cycle, glyoxylate bypass, and transamination were described in our previous works [1, 19].

The activity of glutamate decarboxylase (EC 4.1.1.15) was determined in a reaction mixture (3 ml) containing the following: KH_2PO_4, 50 mM (pH 7.0); glutamate, 20 mM; pyridoxal-5-phosphate, 10 μM; aminooxyacetate (a transamination inhibitor), 5 mM; and cell-free homogenate as an enzyme preparation. Incubation was carried out at 37°C for 60 min under continuous stirring. The reaction was stopped by adding 5% trichloroacetic acid. The supernatant obtained after centrifugation (10000 *g*, 10 min) was examined in an amino acid analyzer. GDC activity was calculated by the rate of γ-ABA production or by glutamate diminution [6].

The activity of 4-aminobutyrate:2-oxoglutarate aminotransferase (EC 2.6.1.19) was analyzed in a reaction mixture containing the following: potassium phosphate buffer, 100 mM (pH 8.2); EDTA, 0.2 mM; KGA, 7.5 mM; 4-ABA, 7.5 mM; NAD, 0.2 mM; and cell-free homogenate. The final volume of the reaction mixture was 3 ml. NADH production was registered at 340 nm [4].

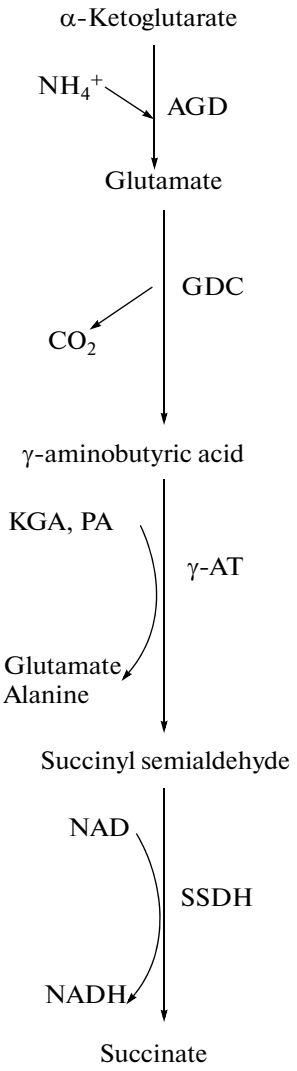

Fig. 1. The scheme of succinate synthesis from glutamate and γ-aminobutyric acid. AGD, aminating glutamate dehydrogenases; GDC, glutamate decarboxylase; γ-ABA AT, γ-ABA aminotransferase (EC 2.6.1.19); SSDH, NAD-dependent succinyl semialdehyde dehydrogenase (EC 1.2.2.16); KGA, α-ketoglutaric acid; and PA, pyruvic acid.

The activity of succinyl semialdehyde dehydrogenase (EC 1.2.1.16) was analyzed in a medium containing potassium phosphate buffer, 100 mM (pH 8.2); EDTA, 0.2 mM; HAD, 0.2 mM; succinyl semialdehyde (SSA), 0.1 mM; and cell-free homogenate. The reaction was started by adding SSA. NAD reduction was measured at 340 nm [4]. The activities of other enzymes in Table 1 were determined by the methods described in [16, 19].

Activities of the enzymes that function during active KGA synthesis in *Y. lipolytica* cells

Enzyme	Specific activity, μmol/min per mg protein
Glutamate decarboxylase	0.09
γ-ABA aminotransferase	0.29
NAD-succinyl semialdehyde dehydrogenase	0.17
Succinate dehydrogenase	0.34
Glutamate dehydrogenase (NADH), aminating	0.23
Glutamate dehydrogenase (НАДФН) aminating	0.14
Alanine aminotransferase	0.25
Aspartate aminotransferase	0.17
Pyruvate dehydrogenase	0.002
Citrate synthase	0.95
NAD-isocitrate dehydrogenase	0.32
NADP-isocitrate dehydrogenase	0.24
KGA dehydrogenase	0.02
Malate dehydrogenase	50.41
Fumarase	0.75

RESULTS AND DISCUSSION

KGA excretion and the pool of free amino acids. Figure 2a shows the dynamics of *Y. lipolytica* culture growth on ethanol under thiamine deficiency and $pO_2 = 50\%$ with a single addition of $(NH_4)_2SO_4$ as a nitrogen source. It can be seen that the culture growth is accompanied by decrease in the nitrogen source concentration below 20–25 mM; under these conditions, KGA synthesis is rather weak. Additional introduction of the nitrogen source (Fig. 2a) resulted in intensification of the synthesis of this product, but it also gradually ceased as the NH_4^+ concentration decreased below the critical value (1–2 mM).

On the contrary, maintaining the nitrogen source concentration at a high level by its fractional, repeated introduction into the medium resulted in a sharply increased KGA excretion (Fig. 3a). This finding indicated the need for a certain high level of nitrogen for the development of adaptive reactions, first of all, those coupled with KGA synthesis and maintenance of the high intracellular level of free amino acids. At the same time, the medium contained no free amino acids.

Qualitative analysis of free amino acids in the cells showed the presence of 17 amino acids that could be incorporated into proteins and one acid that was not a component of these polymers, γ-aminobutyric acid. It should be noted that this broad spectrum of free amino acids did not include aspartic acid, which is a necessary component for the functioning of aspartate aminotransferase (ASP-aminotransferase)involved in one of the most widespread transamination systems of eukaryotic cells. At the same time, the culture medium contained no free amino acids.

The study of the quantitative composition of free amino acids in yeast cells showed that alanine, glutamate, and γ-aminobutyric acid were present in the highest concentrations: depending on cultivation conditions, their concentrations reached 20–40 mg per 1 g of dry biomass. The contents of other acids varied from 2 to 10 mg/g dry biomass.

Concentrations of all free amino acids depended on the period of cultivation: their maximum accumulation was observed in the phase of thiamine-limited growth on ethanol, which confirms our suggestion of the involvement of amino acids in stress response. With a single additional introduction of the nitrogen source (Fig. 2b) during transition into the phase of growth suppression caused by NH_4^+ depletion, the concentration of amino acids abruptly decreased. During this period of culture growth, amino acids can probably be used as a nitrogen source: KGA synthesis ceases as well (Fig. 2a).

On the contrary, with a constant concentration of ammonium ions maintained in the medium (Fig. 3), although the maximum accumulation of free amino acids in cells was observed in the same period of culture development, the amino acid concentration remained practically unchanged and KGA excretion even increased.

Thus, the results show that KGA synthesis in yeast cells on ethanol under thiamine limitation was accompanied by formation of a large pool of free amino acids in the cells. Additional nitrogen limitation caused by decrease in the concentration of NH_4^+ ions resulted in an abrupt (twofold) decrease of both KGA synthesis and consumption of the pool of cellular free amino acids.

Production of succinate from glutamate and γ-ABA. As was noted above, alanine, glutamate, and γ-ABA comprise a significant part of the pool of free amino acids in the cell. This provided additional grounds for believing that high concentrations of these compounds during KGA synthesis were associated with the change in oxidative metabolism of the culture during cultivation on ethanol under thiamine deficiency.

As is known, the active Roberts' bypass in the neural and muscular tissues [20] supports rapid production of succinate from glutamic acid catalyzed by 4-aminobutyrate-aminotransferase (γ-aminobutyrate bypass) (Fig. 1), avoiding the slower stage via KGA. We have suggested that this sequence of reactions resulting in rapid succinate synthesis functions during KGA synthesis from ethanol by the culture under study [1]. Our suggestion was based on the detection of: (a) inhibition of glutamate oxidation [1] by malonate (the inhibitor of succinate dehydrogenase) and aminooxyacetate (the inhibitor of transamination

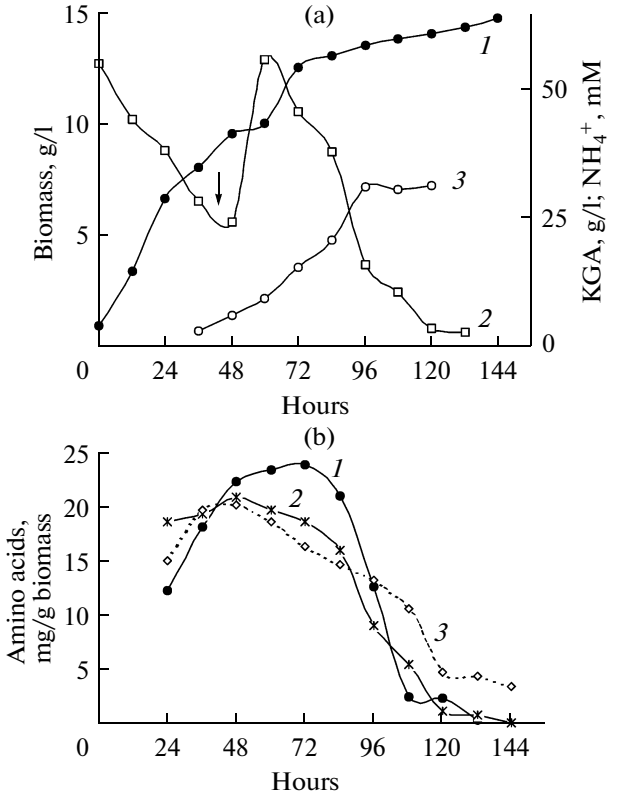

Fig. 2. Growth dynamics (a) and content of amino acids in the cells (b) during yeast cultivation on ethanol. (a) growth dynamics (*1*), nitrogen concentration (*2*), and KGA accumulation in the culture liquid (*3*). Single addition of $(NH_4)_2SO_4$ to the level of 40–45 mM is marked by an arrow. (b) glutamate (*1*), alanine (*2*), and γ-ABA (*3*).

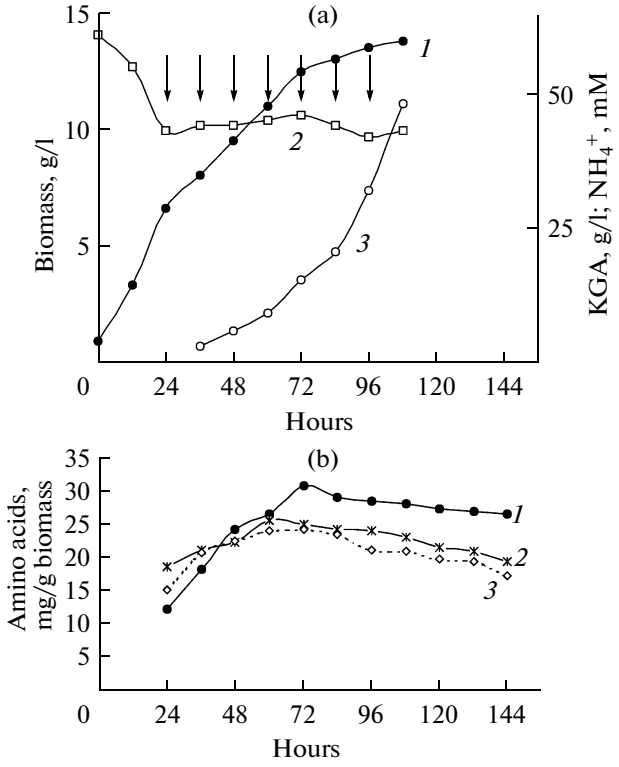

Fig. 3. Growth dynamics (a) and content of amino acids in cells (b) during yeast cultivation on ethanol at a constant $(NH_4)_2SO_4$ concentration. (a) growth dynamics (*1*), nitrogen concentration (*2*), and KGA accumulation (*3*) in the culture liquid. Arrows mark addition of $(NH_4)_2SO_4$ to the level of 40–45 mM. (b) glutamate (*1*), alanine (*2*), and γ-ABA (*3*).

enzymes), (b) high concentrations of glutamate and γ-ABA in the cells during KGA synthesis (see the previous section), and (c) low activity of α-ketoglutarate dehydrogenase (KGA-DH) in conditions of thiamine limitation and high succinate dehydrogenase activity (table). Indeed, with the noted low KGA-DH activity, the subsequent production of succinate would be insignificant. However, high activity of succinate dehydrogenase indicates the existence of a powerful source of succinate production. Such a source may be an isocitrate lyase reaction resulting in the formation of not only succinate, but also glyoxylate necessary for the malate synthase reaction. However, the previously obtained results show that the activity of enzymes of the glyoxylate cycle during the period of KGA synthesis is insignificant [19]. Analysis of the enzymes participating in succinate synthesis from glutamate (glutamate decarboxylase, γ-ABA-transaminase, succinyl semialdehyde dehydrogenase) in cell homogenates of the yeast under study showed their high activity (table).

Thus, it may be concluded that, during KGA synthesis from ethanol under thiamine deficiency, the orthodox TCA cycle may function in a truncated variant: production of glutamate from KGA followed by its decarboxylation and formation of γ-ABA (Fig. 1).

We think that the functioning of the γ-aminobutyrate bypass is an adaptive reaction of a cell to emerging thiamine deficiency, which allows it to avoid a "bottleneck" in the TCA cycle: the thiamine-dependent enzyme ketoglutarate dehydrogenase.

The functioning of enzymes of nitrogen metabolism. The γ-aminobutyrate bypass (Roberts' cycle) needs intense glutamate synthesis for its functioning. Glutamate is known to be formed mainly of KGA and ammonium ions in the reactions catalyzed by NAD(P)H-dependent aminating glutamate dehydrogenases (GDs). Analysis of the activities of aminating GDs (table) demonstrated that NADH-dependent GD played the key role in glutamate production during KGA synthesis from ethanol, whereas the activity of NADPH-dependent GD decreased by the beginning of synthesis. It should be noted that, during cultivation on a complete medium, on the contrary, the activity of NADPH-dependent GD is 3–3.5 times higher than that of NADH-dependent GD [1]. This probably explains the need to maintain the high con-

centration of NH_4^+ ions during KGA synthesis by the culture under study [15].

Thus, a typical feature of metabolism of the studied yeast is redistribution of glutamate dehydrogenase activities depending on the cultivation conditions on ethanol: the activity of NADPH-dependent enzymes prevails during unlimited growth, while transition to KGA synthesis and the activity of NADH-dependent GD occurs during unbalanced growth. It is a manifestation of one more adaptive mechanism of the yeast under nonoptimal cultivation conditions.

It should be noted that the efficiency of KGA synthesis from ethanol at a low concentration of NH_4^+ ions does not depend only on high activity of glutamate dehydrogenases. Our study revealed an association of KGA excretion with enhancement of the activities of aspartate and alanine transaminases (ASP- and ALA-aminotransferases, respectively) and γ-ABA transferase (table) in the yeast cells. The high activities of these aminotransferases under thiamine limitation indicate the essential role of the transamination reaction and, in particular, of the malate–aspartate bypass during KGA synthesis.

Synthesis of ketoacids needed for the functioning of transaminases. Increase of aminotransferase activities under conditions of KGA synthesis from ethanol must be ensured by the high rate of generation of ketoacids, i.e., oxaloacetate (OAA), pyruvate (PA), and KGA, on the one hand, and glutamate, aspartate, alanine, and γ-ABA, on the other hand.

The high cellular content of free amino acids, i.e., glutamate, alanine, and γ-ABA (but not aspartate), during KGA excretion was noted above. At the same time, the processes or enzymes participating in PA and OAA synthesis during growth on ethanol, especially under conditions of thiamine limitation, are not quite clear.

Indeed, aerobic growth of yeast on ethanol is characterized by complete absence of glycolysis, which is considered to be the main source of PA. Direct production of pyruvate from C_2 units of ethanol through carboxylation of acetyl-CoA catalyzed by pyruvate synthase [21] has not been revealed in yeasts. It is believed that acetyl-CoA carboxylation results in malonyl-CoA production, while condensation of two acetyl-CoA molecules leads to the synthesis of acetoacetate.

In our experiments during the growth on ethanol, especially under conditions of thiamine deficiency, acetyl-CoA was formed not of pyruvate (the pyruvate dehydrogenase activity is actually absent under thiamine limitation, see Table), but directly from acetate via acetyl-CoA synthetase reactions. Intense synthesis of KGA (50 g/l) from ethanol [1] is always accompanied by excretion of PA (1–1.5 g/l) into the cultivation medium. Upon introduction of malonate (the inhibitor of succinate dehydrogenase) into the cultivation medium, PA excretion substantially increased (up to 10 g/l). It demonstrates that at least some part of PA is produced after the point of malonate inhibition (succinate dehydrogenase), i.e., is either due to the functioning of the left part of TCA cycle or is not associated with the latter at all.

However, neither pyruvate decarboxylase (EC 4.1.1.1) nor pyruvate oxidase (EC 1.2.3.3), being thiamine-dependent enzymes, can function under severe limitation of this vitamin. The only known enzyme capable of direct reductive carboxylation of acetyl-CoA and CO_2 with PA production is ferredoxin-dependent pyruvate synthase (EC 1.2.7.1) functioning in some plants and bacteria [21]. It should be mentioned that the presence of ferredoxin was noted in the cell spectra of the yeast *Y. lipolytica* during cultivation on ethanol under thiamine limitation [16].

It may be supposed that, before reaching a severe thiamine limitation in the course of cultivation, PA or OAA synthesis proceeds via reductive (with the involvement of NADH) carboxylation of acetyl-CoA (pyruvate synthase).

The resultant gradual accumulation of the high intracellular pool of KGA leads to induction of the enzymes of the γ-aminobutyrate bypass. At the same time, large amounts of OAA and pyruvate are produced due to the functioning of MDG, including decarboxylating ones. The functioning of decarboxylating MDG (EC 1.1.1.38) resulting in direct formation of pyruvate from malate has been shown previously in this yeast species in the study of citric acid and KGA synthesis from ethanol [22].

Detection of significant PA concentration in the culture medium during KGA synthesis from ethanol by this culture suggests that its concentration in yeast cells may be significantly higher or at least the same. This notion is supported by the high content of free alanine in the cells of this yeast strain (Fig. 2), which can be formed only from PA in the transamination reaction with glutamate (ALA-transaminase). Let us note that, in contrast to alanine, neither cells nor the culture medium showed any appreciable quantities of aspartate, although the activity of ASP-aminotransferase was rather high (table). We assume that such a situation is a consequence of the OAA deficiency that developed during monopolization of the pool of this intermediate by citrate synthase and malate dehydrogenase, the activities of which were much higher compared to ASP-aminotransferase (table).

Summing up the research results, one may conclude that cultivation of the yeast *Y. lipolytica* on ethanol under thiamine deficiency causes adaptive stress changes. The increase of ammonium nitrogen consumption and KGA excretion manifest physiological changes, the functioning of the γ-aminobutyrate bypass and the high MDG activity are manifestations of metabolic changes, and the increased activities of transamination reactions (ALA- and ASP-aminotransferases) reflect changes in nitrogen metabolism.

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