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Glutamine Metabolism and Cycling in *Neurospora crassa*

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INTRODUCTION

The nitrogen metabolism of unicellular and multicellular organisms cycles nitrogen through the ecosystem (55, 65). Atmospheric N_2, after being reduced to ammonium, is converted to organic nitrogen and again to ammonium; ammonium may also be oxidized to nitrate and then denitrified to the original N_2. Microorganisms are always engaged in search, fixation, transport, catabolism, assimilation, distribution, storage, and incorporation of nitrogen atoms. In turn, microbial degradation releases ammonium and CO_2. It is during the assimilation of ammonium into glutamine when all these processes converge. Regardless of the form in which the cell acquires nitrogen, ammonium is released, assimilated into glutamate and glutamine, and subsequently used to synthesize the metabolites and macromolecules necessary for cell growth. Ammonification, the production of ammonium during biomass degradation, recycles assimilated nitrogen (64, 65) (Fig. 1).

The synthesis of glutamine is driven by carbon skeletons, reducing power, and ATP. Glutamate can be synthesized by glutamate dehydrogenase (GDH) in a reductive (NADPH-dependent) amination, in which ammonium and 2-oxoglutarate are the substrates (65). Glutamate can also be synthesized in a reaction catalyzed by glutamate synthase (GOGAT), by which two molecules of glutamate are formed as products of reductive transamidation of the ω-nitrogen of glutamine to 2-oxoglutarate (96). Because two molecules of glutamate are formed in this reaction, one molecule is available to synthesize glutamine again; all other transamidases stoichiometrically convert glutamine to glutamate by transferring the amide N to various receptors. The synthesis of glutamine is carried out by the only amide synthetase found in living organisms: glutamine synthetase (GS), which incorporates ammonium into the γ-carboxyl of glutamate after being activated by ATP (59) (Fig. 1). Interconvertion of glutamine and glutamate is also mediated by glutaminases. Thus, glutamine is a central compound in nitrogen metabolism; it regulates the flow of this essential element. Glutamine can be considered the final product of nitrogen assimilation. It donates its nitrogen by irreversible transamidations, in contrast to glutamate, which donates by reversible transaminations. It is therefore a metabolic advantage for glutamine to regulate nitrogen fixation, transport, catabolism, and assimilation. In addition, the final products of distributed glutamine nitrogen regulate GS allosterically and, in consequence, the synthesis of glutamine (27, 90) (Fig. 1).

Nitrogen metabolism at the cell level and nitrogen cycling in the ecosystem are linked with the metabolism of carbon, because energy is expended in glutamine synthesis and carbon skeletons are trapped in the form of organic nitrogen. In addition, nitrogen distribution drains carbon and energy for biosynthetic processes.

Although glutamine metabolism in microorganisms has been exhaustively studied (66, 76), the operation of a glutamine cycle has not been reviewed. The aim of this review is to summarize the operation of the glutamine cycle in the fungus *Neurospora crassa*, in particular, glutamine synthesis leading to assimilation and degradation; characterization and regulation of the enzymes that mediate glutamine cycling; evaluation of energy expenditure by glutamine cycling; and the physiological implications of the glutamine cycle.

GLUTAMINE TURNOVER AND NITROGEN DISTRIBUTION IN *N. CRASSA*

First, I present and discuss the original findings about the turnover of protein and small metabolites, such as glutamine, under conditions where *N. crassa* is limited by an essential nutrient, in the postexponential phase of growth, or when an inhibitor of protein synthesis is present in the medium.

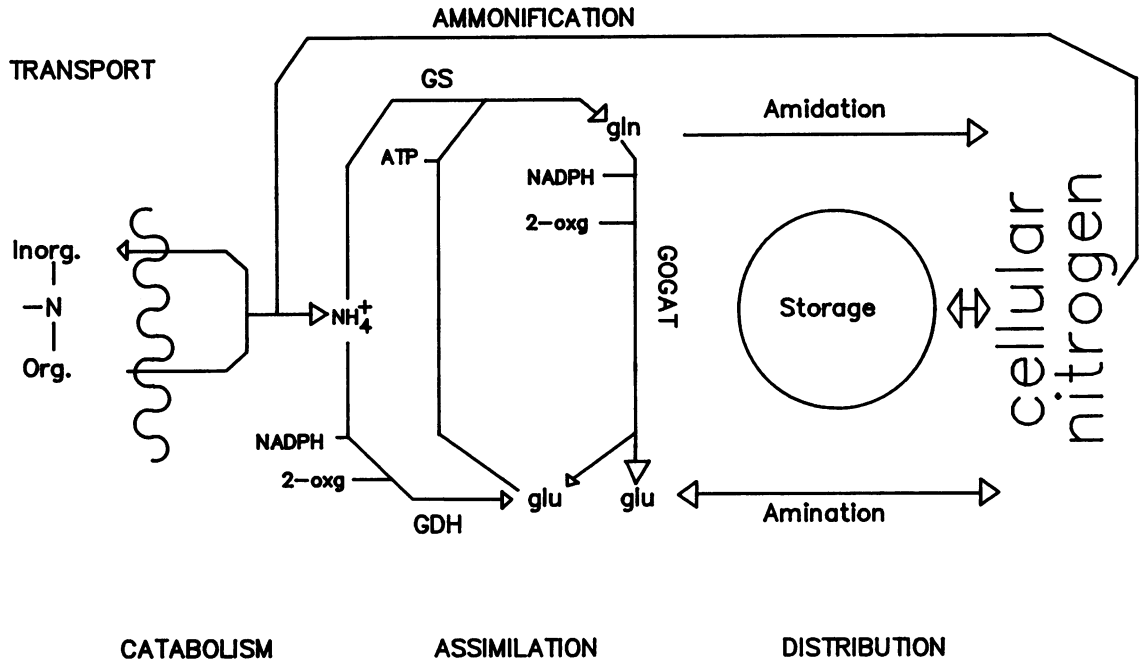

FIG. 1. Schematic representation of nitrogen cell metabolism. Abbreviations: glu, glutamate; gln, glutamine; 2-oxg, 2-oxoglutarate.

Absence of Nitrogen

In the absence of nitrogen, the conidia of an *N. crassa* wild-type strain germinate, increase their dry weight, and form germ tubes. Although the net protein content remains unchanged, de novo protein synthesis occurs (21). Furthermore, the rise of GS activity during nitrogen deprivation is a consequence of this synthesis (21).

In nitrogen-deprived *Klebsiella aerogenes*, a gradual decrease in the level of proteins and in some enzymes is observed. In contrast, the level of GS and other proteins increases as a result of specific synthesis, even if GS was being degraded at the same time (26). The most favored interpretation of why GS-specific activity increases in the absence of nitrogen in several microorganisms is that the cell remains in a highly reactive state with respect to transient changes in the extracellular supply of nutrients (95).

We found that glutamine was synthesized in *N. crassa* in the absence of nitrogen. A glutamine auxotroph (*gln-1b*) accumulated glutamate under these conditions (21, 63). Then, during protein turnover in the absence of nitrogen, active GS was synthesized at a higher rate (21, 63). It is possible that some of the glutamine synthesized in the absence of nitrogen is destined for cell wall formation, as judged by the increase in dry weight (63) and the abundant hyphae devoid of cellular content, which were less apparent in the case of the glutamine auxotroph (*gln-1b*) (see below). It was found that GS also has a role in the formation of glutamine for protein synthesis during cell turnover in the absence of nitrogen (63). After total GS inhibition by the specific inhibitor methionine sulfoximine (MS), protein synthesis in an amino acid auxotroph (*leu-3*) was measured by monitoring [^{3}H]leucine incorporation into protein (63). Ten minutes after addition of MS, no GS activity was detected. The glutamine content decreased to very low levels, and glutamate levels increased slightly. An 80% inhibition in the rate of protein synthesis was observed after GS inhibition (63) (see below). The ammonium flowing through glutamine most probably is derived from cell turnover. Thus, macromolecules and their precursors are interconverted; glutamine is degraded to its carbon skeleton and ammonium and resynthesized. Two points are relevant to this argument: (i) nitrogen-deprived bacterial cultures oxidize carbon and expend energy similarly to cultures growing in carbon and nitrogen excess (97), and (ii) $^{13}NH_4^+$ isotope studies on *Bacillus megaterium* and *Escherichia coli* have shown that a short period of nitrogen deprivation may result in the generation of a large amount of endogenous ammonium (45). If GS and its product, glutamine, are subjected to turnover in the absence of nitrogen, this cycling along with that of other biomolecules must account for the large amount of energy expended by nutrient-deprived cultures (106) (see below).

Growth Restriction

When deprived of certain amino acids in the presence of ammonium as a nitrogen source, *N. crassa* accumulates glutamine and arginine (20). Other conditions that restrict the growth of mycelia, for example, the presence of cycloheximide or transition to the stationary phase of growth, also cause glutamine and arginine to accumulate. A large proportion of the accumulated arginine is sequestered in an osmotically sensitive compartment (68), probably the vacuole first reported and studied thoroughly by Davis and co-workers (17, 18, 93, 105). We reported that glutamine stopped the catabolism of exogenous arginine by inhibiting the induction of arginase (101). We also showed that arginase was not induced by exogenous arginine if the mycelia are deprived of an amino acid (68). Thus, under growth-restricted conditions, the catabolism of nonsequestered arginine, either from biosynthesis or from the medium, was prevented by glutamine.

The regulation of arginase by nitrogen metabolites is supported by the following data: (i) mutations that impair either the formation of ammonium from urea (*ure-1*) or the synthesis of glutamate (*am-1*) result in a higher induction of arginase, when arginine is used as nitrogen source (101); (ii) glutamine, or glutamate plus ammonium, interferes with

arginase induction in the wild-type strain, as well as in the *ure-1* and *am-1* mutant strains (101); (iii) the rates of accumulation of [*guanido*-^{14}C]arginine in the double auxotroph *arg-5 prol-3* are similar, regardless of whether glutamine is present in the medium (however, growth and $^{14}CO_2$ liberation are observed only in the absence of glutamine [101]); and (iv) a regulatory mutant (*gln*^{r}), in which GS and arginase are resistant to repression by glutamine, has been isolated (29). There is a report which shows that arginase can be induced in the presence of ammonium (22). Since the growth conditions of *N. crassa* in that work (22) were different from ours (101), any comparison is difficult (17). In addition, glutamine must play an important role in the accumulation of arginine into the vacuole for the following reasons: (i) in contrast with the wild-type strain, the *gln*^{r} mutant accumulated substantial amounts of arginine in the vacuole and glutamine in the cytosol while growing exponentially (29); (ii) when incubated on minimal medium, amino acid-auxotrophic mutants accumulated glutamine and arginine (20, 68); (iii) nitrogen starvation, perhaps signaled by glutamine depletion, causes the accumulated arginine to be released from the vacuole (29, 51); and (iv) the accumulated glutamine prevents arginase induction (68). These data have led us to conclude that during unrestricted growth, endogenous glutamine regulates the accumulation of arginine into the vacuole; however, during restricted growth or in the presence of the *gln*^{r} mutation, glutamine regulates neither its own synthesis nor the accumulation of arginine by the vacuole (20, 29, 50).

As described above, in the absence of nitrogen, glutamine turnover has an important role in protein turnover. The finding that under nongrowth conditions but in the presence of nitrogen the synthesis of glutamine was required to distribute its nitrogen also seemed paradoxical. Thus, in strains with mutationally impaired GS activity (16), the accumulation of arginine and glutamine that occurs in the presence of glutamine was not observed; instead, glutamate accumulated (21). These observations can be explained by assuming that a glutamine cycle operates under growth-restricted conditions. The first step of this cycle is transamination of the α-amino group of glutamine, thus forming an amino acid and 2-oxoglutaramate; in the second step 2-oxoglutaramate is hydrolyzed to 2-oxoglutarate and ammonium by an ω-amidase; in the third step, glutamate is formed by GDH; and in the last step, glutamine is synthesized by GS (21). This glutamine transaminase-ω-amidase pathway has been studied in great detail in animal cells (11), and the *N*-transaminase of glutamine was found in *N. crassa* a long time ago (61). In addition, the GDH of this fungus has been studied in depth by Fincham (23) (see below).

There are indications that glutamine is also cycled during growth. An asparagine auxotrophic mutant of *N. crassa* is able to grow when supplied with 2-oxosuccinamic acid (the 2-oxoacid of asparagine) and ammonium, a fact that was explained by the activity of a glutamine transaminase (61). As noted above, a nitrogen-regulatory mutant strain (*gln*^{r}), in which GS is only partially repressed by glutamine, accumulates glutamine and arginine while growing exponentially in ammonium or glutamine as the nitrogen source (29). In this way the *gln*^{r} strain resembles nongrowing mycelia with respect to amino acid accumulation (20, 68). In addition, this mutant has a high capacity to accumulate arginine even when ammonium is supplied only in limited amounts (29). The evidence indicating that glutamine cycles in the *gln*^{r} mutant strain during growth is that the additional presence of an *am-1* (GDH-negative) or *gln-1b* (GS-reduced) mutation reduces arginine accumulation in the presence of glutamine as the nitrogen source (29).

Carbon Restriction

Whereas the level of GS is maintained constant during nitrogen deprivation, it is degraded during carbon source deprivation (67). This degradation is enhanced when glutamine is added (67). Similar results were obtained when carbon-limited fed-batch cultures were used (53). If the mycelium was deprived of carbon and nitrogen, GS activity and protein were degraded and ammonium was excreted. However, if carbon was restored, the GS concentration increased, and the excreted ammonium was reassimilated and incorporated into protein (67). The preferential degradation of GS prevents the synthesis of glutamine, so carbon and energy are spared for cell maintenance (67). More importantly, in the absence of carbon and nitrogen, cell biomass is degraded. During this process GS and other proteins are degraded and ammonium is released, allowing the utilization of carbon skeletons. These molecular events are reversed by addition of a carbon source (67). It is clear that biomass degradation, including ammonium liberation, is to some degree a reversible process, because cell growth can be reinitiated.

GLUTAMINE SYNTHESIS IN *N. CRASSA*

GS: Differential Monomer Expression and Regulation of Enzyme Activity

With the exception of rhizobia, in which two GSs participate in the assimilation of ammonium (25), procaryotic organisms produce only one GS, composed of identical subunits (90). In some eucaryotic organisms, two different subunits make up the GS octamer. This is the case for *Chlorella kessleri* (94), plants (48, 80), *Drosophila melanogaster* (5), and Chinese hamster cells (32). Purified preparations of *N. crassa* GS also contain two nonidentical subunits. These monomers can be separated by acrylamide gel electrophoresis in sodium dodecyl sulfate-urea gels, in which one of them (GSα) runs slightly slower than the other (GSβ) (73, 83). Evidence from mRNA separation in agarose gel electrophoresis and in vitro translation indicated that different mRNAs code for α and β subunits (47). Additional evidence suggests that the subunits are encoded by separate genes: a mutation confering auxotrophy due to an altered β subunit (14, 15) segregated independently of a mutation that affects the catalytic activity of the α subunit (6a).

In the presence of excess exogenous carbon and nitrogen, glutamine represses the octameric GSβ enzyme which is induced by glutamate in the wild-type strain (63, 103). Glutamine regulates the synthesis (77), specific mRNA concentration (47), and degradation (67) of GS. In the postexponential phase, the synthesis of GSα is also induced, and octameric GS hybrids composed of α and β monomers are found (47). When the cultures are transferred from ammonium excess to ammonium limitation, the synthesis of the octameric GSβ enzyme is greatly diminished; the α polypeptide is synthesized and GS activity is associated with a tetramer (15, 47). The activities of octameric GSβ and tetrameric GSα are different (54, 67). Purified preparations of these enzymes show that in the presence of Mg^{2+} or Mn^{2+}, the tetrameric GSα has 12- to 70-fold-lower activity than the octameric GSβ (J. Guzmán and J. Mora, unpublished data). A low in vivo activity of the GSα tetramer is reflected in a lag

phase and suboptimal growth of a mutant (*gln-1bR8*) that lacks β subunits. This mutant strain contains a high-glutamate pool that partially compensated for the low GS activity (54). It is puzzling that cultures shifted from high to low ammonium synthesize more GSα than GSβ. As will be further described below, a high GS activity together with the cycling of glutamine can drive a high carbon flow and even deplete the cell of glutamate and energy. Therefore, it is possible that the role of α monomers is to modulate the activity of the GSαβ hybrid oligomers and adjust the cell to slow-growth conditions. Since the tetrameric GSα has a very low activity, the GSα monomers might dilute GS activity by substituting for GSβ monomers in a hybrid GS enzyme. In this regard, mutants with altered β monomers (*gln-1b*) form oligomeric hybrids with the wild-type α monomers that have 20- and 5-fold less GS activity than the GSβ and the GSα enzyme, respectively (14, 16, 54). This inhibition may be an example of how the GSβ catalytic activity can be decreased and how altered β monomers can act as inhibitors of GSα activity. In contrast, it was not possible to obtain mutants in which altered GSα monomers inhibit GSβ activity (14, 16). Only GSα mutants modified in their catalytic activity were found. If the GSα monomers were selected as negative modulators of GS activity, it is very unlikely that a mutant form of the monomer would affect GSβ activity (6a).

As has been reported for *E. coli* GS (52, 71), oxidation of the *N. crassa* α and β polypeptides inactivates the enzyme, giving rise to two acidic polypeptides which are more susceptible to proteolysis than the unmodified species (1). Oxidative modification of GS might be another mechanism for regulating this activity.

Among the allosteric inhibitors of GS activity are amino acids such as alanine, serine, and glycine (27). Since the nitrogen in these amino acids does not come from glutamine, their effect on GS activity is not easily understood. We studied the effect of exogenous amino acids on GS activity and observed a preferential induction of GSβ synthesis over GSα synthesis when glycine or serine was present in *N. crassa* cultures (35). The inhibitory effect of these amino acids on glutamine synthesis is an example of the regulation of glutamine synthesis by α-amino nitrogen (35). Initially, GS inhibition would favor ammonium assimilation into glutamate and other amino acids; however, by reducing the glutamine pool, GS synthesis would be induced, stimulating glutamine synthesis. This mechanism regulates the assimilation of ammonium into glutamate or glutamine (35).

Role of GDH and GOGAT

The role of GDH and GOGAT in *K. aerogenes* is to provide glutamate for glutamine synthesis under high- and low-ammonium conditions, respectively (3). The GS-GOGAT pathway allows the cell to assimilate ammonium from low exogenous concentrations owing to the high affinity of GS for this compound (41, 60). In contrast, the GDH-GS pathway functions more rapidly, but only with higher exogenous concentrations of ammonium because of the low affinity of GDH for ammonium (109). However, it is common for GDH and GOGAT to operate at the same time in other microorganisms (41, 85). It was demonstrated that the main supply of glutamate in *N. crassa* is through GDH with a high exogenous ammonium concentration; even when the supply of this compound is limited, a significant amount of glutamate is synthesized by GDH (54). Experiments using chemostat, ammonium-limited cultures suggested that GDH and GOGAT activities both contribute to glutamate synthesis under such conditions (54). A GOGAT-negative mutant strain (*en-am-2*) altered in the structural gene (39, 81) accumulates large amounts of glutamine in chemostat, ammonium-limited cultures (54). Decreasing the concentration of exogenous ammonium reduces the fraction of glutamate synthesis via GDH; however, owing to its low K_m for ammonium, GS would convert this amino acid to glutamine and then GOGAT (which is derepressed under these conditions) would compensate for glutamate utilization by GS (54). Under conditions of ammonium excess, a GOGAT-negative mutant has slightly lowered glutamate pools and increased glutamine pools, resulting in GS repression (54). Thus, part of the function of GOGAT is to return to glutamate some of the nitrogen incorporated into glutamine by the GDH-GS ammonium assimilation pathway. Accordingly, glutamate represses GOGAT and GDH activities (36, 38, 39, 54). It should be mentioned that almost all glutamine is found in the soluble fraction of the cell (29, 54).

Studies by Kanamori et al., using in vivo ^{15}N nuclear magnetic resonance spectroscopy, confirm what has been reported here about how glutamine synthesis is regulated when suspensions of *N. crassa* mycelia are exposed to ammonium and various amino acids including glutamine (40).

From studies on *N. crassa* mutants (*gln-1bR8* [GSα], *gln-1b* [GSβ negative], *am-1* GDH negative], and *en-am-2* [GOGAT negative]), the following relations between GSα, GSβ, GDH, and GOGAT activities have been revealed (54). When glutamine is synthesized only by tetrameric GSα (*gln-1bR8*), a large amount of glutamate (synthesized by GDH) is required to support growth. If GOGAT is the only supplier of glutamate (GDH negative), the presence of octameric GSβ is necessary to provide glutamine in adequate amounts. A mutant strain which has only the GSα-GOGAT pathway cannot assimilate ammonium, as shown by its lack of growth in the presence of ammonium excess (54).

The fact that glutamine does not completely repress GS synthesis in wild-type *N. crassa* during growth (47, 103) opens the possibility of the existence of glutamine cycling under these conditions.

GLUTAMINE ASSIMILATION AND DEGRADATION IN *N. CRASSA*

The conversion of glutamine to glutamate by GOGAT, which has been shown to be present in *N. crassa* (38, 39), together with the catabolism of glutamate by NADH-dependent GDH, would release 2-oxoglutarate and ammonium. As shown below, this pathway operates when glutamine is utilized as a carbon and nitrogen source and when glutamate and glutamine synthesis is impaired by mutation (8).

In contrast, in nitrogen-limited chemostat cultures, GDH supplies the glutamate that is converted to glutamine, and the role of GOGAT is to return some of this nitrogen to glutamate (54). In this way, a glutamate-glutamine cycle fed by exogenous ammonium operates in the wild type. A mutant lacking GOGAT accumulated very high glutamine levels, indicating that the presence of GOGAT allows *N. crassa* to grow in the presence of a high concentration of glutamate and a low concentration of glutamine (53, 54, 63). However, it is important to maintain the concentration of these amino acids within a certain range. In ammonium-limited chemostat cultures, a mutant strain that lacks β polypeptides and synthesizes glutamine by using only the tetrameric GSα accumulated excessive amounts of gluta-

TABLE 1. Distribution of label derived from L-[*amide*-^{13}N]glutamine among various metabolites[a]

Metabolite	Percentage of label in:			
	Wild-type strain		GDH-negative mutant strain (10-min label)	Wild-type strain plus MS[b] (25-min label)
	5-min label	25-min label		
Acid compounds[c]	1.36	2.60	1.34	
Carbamyl phosphate	0.18	1.21	0.19	
Aspartate	0.16	0.26	0.43	
Glutamate	8.34	13.9	16.1	22.7
Glutamine[d]	86.0	78.4	43.4	40.2
Proline[e]	0.37	0.24	0.81	
Ammonium	2.98	0.78	14.4	
Histidine[e]	0.07	0.19	ND[f]	
Arginine	ND	1.21	ND	
Alanine	ND	ND	21.9	14.7
2-Oxoglutaramate	ND	0.07		
Glutamine (amine)	1.73	8.00	5.60	1.7

[a] Reprinted from the *Journal of Bacteriology* (6) with permission.
[b] Incubated previously for 20 min with MS.
[c] Compounds of undetermined structure eluting earliest from the Partisil 10SCX analytical cation-exchange column.
[d] ^{13}N label in amine plus amide.
[e] Tentative assignment.
[f] ND, Not detected.

mate; its growth yield was lower than that of wild-type and other mutant strains (54).

The contribution of other transamidases and glutaminases to the conversion of glutamine to glutamate in *N. crassa* must be minor. This conclusion comes from the observation that a double-mutant strain lacking both GOGAT and GDH grows slowly with glutamine as the nitrogen source. This growth rate is stimulated by alanine or other amino acids that yield glutamate efficiently (7).

It is interesting that a highly active transamidase may constitute an alternate route for the primary assimilation of ammonium. This is the case for a strain of the cyanobacterium *Anabaena* sp. which lacks GDH and GOGAT activity. The presence of DL-7-azatryptophan leads to a severalfold increase in the activity of carbamoylphosphate synthetase (with its corresponding glutaminase activity) in this strain (10).

In cell extracts of *N. crassa*, the concerted action of a glutamine transaminase and an ω-amidase may convert glutamine to 2-oxoglutarate and ammonium, through the formation of 2-oxoglutaramate as an intermediate (9). The following results indicate that this pathway functions in vivo: (i) 2-oxoglutaramate was detected in *N. crassa* grown in the presence of ammonium, or glutamine, as the nitrogen source, its concentration being fivefold higher under the latter conditions (9); (ii) 2-oxoglutaramate was detected only in the presence of 6-diazo-5-oxo-L-norleucine (an inhibitor of amidases), and the absence of 2-oxoglutaramate in the presence of aminooxyacetate (an inhibitor of transaminases) plus 6-diazo-5-oxo-L-norleucine indicated that the transaminase-ω-amidase pathway participated in glutamine degradation (9); and (iii) after the addition of L-[*amide*-^{13}N]glutamine to the wild-type strain, a small but significant amount of label was found in 2-oxoglutaramate (6).

There is a study (88) in which the presence in *Saccharomyces cerevisiae* of both glutamine transaminase and ω-amidase was demonstrated, although it is unknown whether these enzymes operate as part of a glutamine cycle. The presence of two glutaminases was also reported. The authors proposed that glutamine degradation proceeds via the transaminase or via the glutaminase depending on the pyruvate level, because this compound inhibits glutaminase B (89).

GLUTAMINE CYCLING

Following the finding that GS activity is required in *N. crassa* to distribute the nitrogen of glutamine (21), evidence has accumulated indicating that glutamine is cycled in this fungus through the transaminase-ω-amidase pathway: (i) ammonium was excreted to the medium by a double-mutant strain lacking GOGAT (*en-am-2*) and GDH (*am-1*) during growth in glutamine as the nitrogen source, and aminooxyacetate and 6-diazo-5-oxo-L-norleucine inhibited the release of ammonium from glutamine (9); (ii) more ammonium from glutamine was excreted by the double-mutant strains (GO GAT negative, GDH negative; and GDH negative, CrS reduced) than by the single mutant (GDH negative) (7); (iii) a mutant that lacks GOGAT activity accumulated glutamine and had a small amount of glutamate and alanine when grown in glutamine as the nitrogen source (7); (iv) when grown in glutamine as the nitrogen source, a strain with a GS-reduced mutation accumulated more alanine and glutamate than the wild type did (7); and (v) glutamine is labeled when growing in [^{14}C]sucrose and glutamine, and the specific radioactivity was fourfold lower in a strain with a GS-reduced mutation (*gln-1b*) during growth in [^{14}C]sucrose and glutamine (7). The evidence presented above has been reviewed in more detail elsewhere (62).

Recent experiments on the distribution of L-[*amide*-^{13}N]glutamine have demonstrated that the glutamine cycle operates during growth of wild-type *N. crassa* and have given information about the rate and amount of nitrogen cycling (6). After the addition of L-[*amide*-^{13}N]glutamine to the wild-type strain, glutamate was more highly labeled than other nitrogen-containing metabolites. The fact that the labeling of the amino group of glutamine increased linearly during the first 25 min after the pulse (Table 1) provides direct evidence that the amide nitrogen of glutamine was redistributed in the glutamine molecule, either through the formation of glutamate by GOGAT or by the glutamine transaminase-ω-amidase pathway, followed by reductive amination by GDH (NADPH dependent) (6).

Further analysis in the GDH-negative (*am-1*) strain showed that the relative amount of label in ammonium was higher than that found in the wild-type strain (Table 1). In

this mutant, the intracellular ammonium pool was larger and the glutamate pool was smaller than in the wild-type strain (6). Thus, a significant fraction of the ammonium derived from glutamine degradation is normally assimilated by GDH into glutamate. GOGAT activity must be responsible for [^{13}N]glutamate formation in the GDH-negative strain, since a glutaminase would produce [^{13}N]glutamate from L-[*amide*-^{13}N]glutamine only after glutamine had been labeled in the amino group. The labeling of glutamate is threefold higher than that in the amino group of glutamine. Thus, a relatively large incorporation of label into glutamate in the GDH-negative strain can be explained only as a result of high GOGAT activity (54) and the accumulation of 2-oxoglutarate (53, 69). In contrast, label incorporated into glutamate in the wild-type strain is lower than in the GDH-negative strain because glutamate is synthesized to a large extent by GDH. The difference in the labeling of the amine and amide groups of glutamine in the wild-type strain, compared with those of the GDH-negative strain (Table 1), may be understood if one assumes that in this mutant, a fraction of the NH_4^+ accumulated lowers the relative specific activity of [^{13}N]ammonium more than the relative specific activity of [^{13}N]glutamate (6, 54).

The important roles of GDH and GOGAT were also obtained from experiments carried out with a GOGAT-negative mutant (*en-am-2*). After 20 min of incubation with L-[*amide*-^{13}N]glutamine, 83% of the label was recovered in glutamine (from which 90% was in the amide and 10% was in the amine position) and 3% was in glutamate. Thus, 11% of the label in the GOGAT mutant was in glutamine (amine) plus glutamate, compared with 22% in the wild-type strain (6). It is possible that in the GDH-negative strain there was an appreciable transfer of amine nitrogen to alanine as result of pyruvate accumulation (53, 69). However, in the wild-type strain nitrogen derived from glutamine (amide) was not diverted to other compounds, but was rather efficiently incorporated into glutamine (amine) via glutamate (Table 1). Glutamine synthetase inhibition by MS greatly decreased the label in glutamine, caused labeled glutamate to accumulate, and caused some label to be transferred to alanine (Table 1). The retention of more than 90% of the ^{13}N label in glutamate and glutamine in the wild-type strain indicates that almost all the nitrogen was retained by cycling between these two metabolites (Table 1) (6).

It is important to emphasize that glutamine cycling operates continually under a variety of growth and culture conditions; in this way it is similar to protein turnover (74).

E. coli and *Salmonella typhimurium* grow as well with glutamine as with ammonium as a nitrogen source. However, in contrast with ammonium, when glutamine is the nitrogen source, GS synthesis is not repressed (78). The lack of repression of GS by glutamine may lead to glutamine cycling in these organisms.

Glutamine Synthesis, Carbon Utilization, and Energy Expenditure

The operation of a cycle that turns over nitrogen between ammonium, glutamate, and glutamine under conditions where growth is not restricted poses the question of the futility, the advantage, or even the necessity of this cycle. On the other hand, the drainage of carbon skeletons, reducing power, and ATP as result of glutamine cycling is evident. Thus, nitrogen cycling might affect the carbon yield and energy distribution.

Inhibitors of GS can be used to prevent glutamine cycling in mutant strains impaired in glutamate and glutamine synthesis. Amino acids such as glycine and serine completely inhibit the growth in ammonium of an *N. crassa* mutant lacking GDH (23). One such mutant (*am-1*) lacks GDH because its synthesis of glutamate is limited (owing to a low GOGAT activity), and it accumulates 2-oxoglutarate (38, 39, 53). The complete inhibition of growth of this strain by glycine and serine has been attributed to the inhibition of GS by these amino acids under conditions in which glutamate levels are low (35). In the presence of these amino acids or MS, 2-oxoglutarate did not accumulate (34, 69). Similar results were observed in fed-batch, carbon-limited, wild-type *N. crassa* cultures (53). Indeed, the coupled carbon and nitrogen flow in glutamine synthesis was more important for growth than is the absolute glutamine content. When the growth of a mutant of *N. crassa*, affected in the GSβ polypeptide and having 20-fold less GS activity than the wild type, was arrested with glycine or serine, the synthesis of glutamine decreased 100-fold while the content of glutamine decreased only by half (35). These data suggest that the flow of carbon and nitrogen to glutamine is a critical step for cell growth (35).

It was found that restriction in glutamine synthesis and cycling results in an impairment in carbon flow. The effect of GS synthesis on carbon utilization was tested directly by measuring the synthesis of tricarboxylic acid (TCA) cycle intermediates in the GDH-negative (*am-1*) mutant strain, 30 min after transfer to a [U-^{14}C]sucrose-ammonium medium containing glycine or MS. It was observed that the specific radioactivities of 2-oxoglutarate, pyruvate, succinate, and malate were reduced 10-fold in the presence of glycine and less so in the presence of MS (34). The effect of reducing glutamine synthesis and carbon flow was also demonstrated in the GS-reduced mutant growing in the presence of glutamine as the nitrogen source. The CO_2 released from [U-^{14}C]sucrose oxidation was also reduced in the GS-reduced mutant in comparison with the wild-type strain (34). The effect of inhibiting glutamine synthesis was further enhanced when MS was added to the GS-reduced mutant strain and the culture was shifted to glutamine and radioactive sucrose. Under these conditions a higher inhibition of GS activity was observed, with a consequent decrease in synthesis of glutamine, glutamate, and 2-oxoglutarate from sucrose. However, most important, a low growth rate was observed despite a sizable glutamine pool (34). Then, under these conditions, growth was limited by carbon restriction and not by nitrogen limitation (34). In this context, the above-described severe diminution of protein synthesis in nitrogen-deprived cultures after GS inhibition (63) can be explained by a reduction in carbon utilization.

A relation between GS activity, glutamine cycling, and carbon metabolism was demonstrated by using mutant strains that were either resistant or sensitive to glycine. One strain with mutant GSβ (*gln-1k*) was more sensitive to glycine than was the wild type inactivated (35). In this mutant, inactive GSβ polypeptides the low activity from the GSα monomers present in the oligomeric hybrids (14, 35). GDH-negative strains accumulate 2-oxoglutarate and are therefore glutamate limited (69). Cultures of GDH-negative strains increased their ATP levels 15- to 20-fold when glycine or MS was added (62). However, these GS inhibitors prevented the normal, large increase in 2-oxoglutarate levels (34, 69). These results indicate that the inhibition of glutamine synthesis and its cycling impairs carbon flow and ATP utilization. A glycine-resistant mutant (*gly*^{r}) was selected from a strain with a GDH deletion (*am-132a*). Con-

trary to the parental strain, the GDH-negative;gly^r (*am-132a;gly*^{r}) mutant strain did not accumulate 2-oxoglutarate and showed low pyruvate and succinate concentrations when grown in ammonium with or without glycine (69). The GDH-negative;gly^r double-mutant strain was also resistant to serine and MS. All these results indicated that the *gly*^{r} mutation is responsible for a reduced carbon flow and for the resistance to the effect of glycine (69). Several steps in glycolysis and in the TCA cycle are regulated by ATP; therefore, it is possible that the reduced carbon flow of the *gly*^{r} mutation is due to the reduced sensitivity of one of these enzymes to ATP inhibition and loss of some of its activity.

Furthermore, the GDH-negative;gly^r strain had a higher growth rate than the GDH-negative single-mutant strain with or without the inhibitor (69). This was explained by the higher GOGAT activity demonstrated in vivo (69), a consequence of a lower content of the TCA cycle intermediates that inhibit this activity (69). The accumulation of 2-oxoglutarate in the GDH-negative strain indicates that even some synthesis of glutamate and glutamine by the GS-GOGAT pathway drives carbon flow and accumulation (69).

It is hard to understand why glutamine is not a carbon and nitrogen source in *N. crassa*. Glutamine can be converted to 2-oxoglutarate either through the transaminase-ω-amidase pathway or by the action of GOGAT and a catabolic GDH; in either pathway, carbon skeletons are supplied to the TCA cycle. However, the glutamine cycle expends energy and traps 2-oxoglutarate and glutamate, something that limits the supply of these carbon skeletons to the TCA cycle. To prevent this energy drain and make glutamine available for catabolism, we constructed a double-mutant strain (GDH negative, GS reduced) that is partially defective in the synthesis of glutamate and glutamine. Small amounts of these amino acids can be synthesized by the GSα-GOGAT pathway (8, 54). This GDH-negative, GS-reduced mutant strain was able to grow in glutamine as the nitrogen and carbon source (8), probably because it is able to supply glutamate and 2-oxoglutarate to the TCA cycle. In this double mutant the deficiency in GS activity prevented utilization of the glutamate generated by GOGAT activity, so this amino acid can be catabolized to 2-oxoglutarate by the (NAD-dependent) GDH. In addition, the lack of biosynthetic GDH precludes the utilization of 2-oxoglutarate formed by the catabolic GDH or the glutamine transaminase-ω-amidase pathway. A higher ATP, ADP, NADH, and NADPH content was observed in the GDH-negative, GS-reduced strain than in the wild type (8). The considerable difference in chemical energy indicates that the expenditure of ATP by GS and NADPH for glutamate synthesis is very high and that a mutant strain that lacks these enzyme activities can save energy which can, in turn, be used for cell growth.

Futile Cycles

The existence of cyclic processes for various purposes, such as oxidation, synthesis, and generation of energy, is common in living organisms. Futile cycles are those in which forward and reverse reactions, catalyzed by different enzymes, operate simultaneously. In one direction, the reactions expend high-energy compounds. Operation of these cycles dissipates energy (42, 43, 72).

Many futile cycles related to the metabolism of glucose occur in animal cells (42, 43). Other futile cycles in higher organisms include adenosine-AMP (2), triglyceride-fatty acids (4), and glutamate-glutamine (31). The combined action in rat liver of GS and glutaminase (the former a cytosolic enzyme and the latter a mitochondrial enzyme) can effect glutamine transport from one compartment to the other as well as regulate glutamine concentration (31). The cycling of glutamine was established by measuring CO_2 release from [^{14}C]glutamine and glutamine release from the perfused liver (31). On the basis of these studies, an interorgan glutamine cycle has been proposed in which glutamine is synthesized by the liver, exported to other tissues, and then degraded to ammonium. When ammonium returns to the liver, glutamine is resynthesized.

There is still some controversy about whether futile cycles are imperfections in metabolic control, simple metabolic leaks (43), means of improving the sensitivity of metabolic regulation (72, 91), or mechanisms to regulate temperature (43). It has been difficult to demonstrate that futile cycles in microorganisms are associated with glucose metabolism in growing cells (13). However, in one study, heat-induced futile cycling of trehalose in *S. cerevisiae* has been clearly demonstrated (37). A possible function of this cycle is to maintain a constant concentration of glucose and thus to prevent damage caused by a high concentration of this metabolite (37).

More recently, it has been shown that an increasing number of enzymes are regulated by interconversion cycles (28). Covalent modification of an enzyme can significantly alter its activity and its ability to control biological systems. These reversible modifications involve phosphorylation, adenylylation, methylation, and ADP-ribosylation of proteins. In these processes, energy is expended and at least two enzymes are required for the interconversion of the forms of the protein. Dual control provides maximum sensitivity with minimal energy expenditure (28); however, a significant fraction of the total energy of an organism is required for the reactions regulated by covalent modification (28).

In general, it is accepted that during optimal growth of microorganisms, there is little turnover of metabolites and macromolecules. For instance, proline is not turned over during growth of *S. typhimurium* (12). Even glycine betaine, a molecule which is accumulated in response to salt stress by some bacteria, does not turn over; only its synthesis, not its degradation, is controlled (86). However, other molecules such as murein (a central component of the bacteria cell wall [19]), acetyl coenzyme A (102), and NAD (100) are turned over during growth. Murein turnover is required for normal surface expansion (19). A major function for the pyridine nucleotide cycle is the regulation of NAD levels. Other (minor) functions are the entrance of certain pyridines in the cycle that are utilized as a source of NAD. In addition, the cycle is a way of supplying pyridine nucleotide cycle intermediates for use in other pathways (100).

Relevant to this discussion is the turnover of molecules involved in transfer or transport reactions through energy barriers or membranes. Energy is also required to support this turnover, but the important feature of these reactions is the associated change in the local concentrations of the reactants (58, 82).

Physiological Significance of the Glutamine Cycle

The glutamine cycle found in *N. crassa* during nitrogen limitation, growth restriction, or even growth may be understood in different ways.

First, biosynthetic activities are maintained in the absence of nitrogen by recycling of nitrogen. The whole cell is

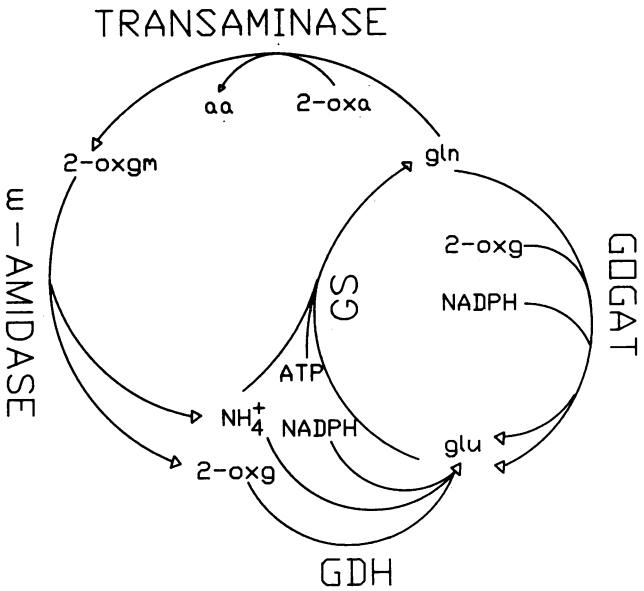

FIG. 2. Schematic representation of the glutamine cycle. Abbreviations: glu, glutamate; gln, glutamine; 2-oxg, 2-oxoglutarate; 2-oxa, 2-oxoacid; aa, amino acid; 2-oxgm, 2-oxoglutaramate.

involved in a grand cycle that consumes energy through scavenging its own nitrogen when nitrogen is not present in the medium. Glutamine cycling is part of the general nitrogen and/or cell turnover, and it has a determinant role in protein turnover; the inhibition of glutamine synthesis and its cycling severely impairs protein synthesis, as mentioned above and discussed below. This may be the result of limiting carbon and energy flow for biosynthetic processes. Under these conditions, the cell can escape and differentiate to sporulate, or it can germinate and explore the medium. As mentioned above, some hyphae appear, devoid of cellular material, which poses the question about biomass reabsorption and/or displacement and its relation with cellular turnover.

The cycling of glutamine and its role in protein turnover, described here for *N. crassa*, may well be related to a recent proposal about the role of glutamine in the regulation of protein turnover in skeletal muscle (104). Glutamine is the most abundant free amino acid in the body and seems to transport carbon and nitrogen between tissues (87). A relation between muscle proteolysis and glutamine production is a well-known fact. Rennie and co-workers proposed that the decrease in the intramuscular glutamine concentration regulates the rate of protein turnover (79). They found that raising the intramuscular glutamine concentration in a rat hindlimb preparation increases the rate of protein synthesis (56) and inhibits protein degradation (57).

Results on ammonium and glutamine assimilation in *N. crassa*, obtained from the study of mutant strains, inhibitors, and labeled compounds (7, 9, 14, 54), led to a proposal of the operation of a cycle involving glutamate, glutamine, and ammonium (Fig. 2). This cycle functions not only in resting cells but also during growth. The only other evidence of nitrogen cycling in microorganisms is a report showing that labeled ammonium is turned over in some bacteria (45). However, in that work (45) it is not clear whether this happens during growth or when the cell is deprived of nitrogen (45). If the turnover of ammonium occurs during bacterial growth, it is possible that the cycling of glutamine, in addition to mobilizing the N-amide of glutamine to glutamate, involves assimilation and redistribution of endogenous ammonium generated from the catabolism of macromolecules. Perhaps, as with protein turnover, this process always exists, but the rate and extent depend on the growth rate and the specific proteins involved (70). Pulse experiments with a variety of nitrogen-labeled compounds, measuring the label incorporated into the N-amide of glutamine, may quantify the turnover under conditions in which the cell is growing optimally. Experiments with ^{15}N labeling in *N. crassa* have indicated that the half-life of glutamine is close to 1 h, but they do not distinguish between exogenously and endogenously generated ammonium (49). To use glutamine as carbon and nitrogen source, *N. crassa* must stop the turnover of this amino acid (8). In contrast to bacteria, in which, under similar conditions, no GS activity is found and glutamine is efficiently channeled for catabolism and oxidation, *N. crassa* is more dependent upon nitrogen turnover, as in the case of high eucaryotic cells (74).

We propose that the most important role of glutamine cycling is energy dissipation. Tempest and co-workers (98, 99) pointed out that an unsolved problem in growth yield and energy distribution is that more energy is obtained from catabolism than is necessary for biosynthetic processes. The difference is not small; it varies between 50 and 200% in cells growing under aerobic or anaerobic conditions (98). Owing to the reactions in carbon catabolism that are regulated by ATP, including respiratory control, it is to be expected that if catabolic reactions are disassociated from anabolism, the operation of an ATPase becomes necessary (98). Other alternatives are to catabolize glucose by pathways that have a low efficiency for ATP generation (98) or even to reduce the number of points of oxidative phosphorylation (98). In relation to the energy expenditure for cell maintenance, the values seem too small to account for the difference. Under conditions of carbon excess, cultures limited by other nutrients often expend energy by catabolic processes uncoupled from anabolism (97). Tempest and Neijssel proposed that a good candidate for an energy-spilling reaction is the synthesis of glutamine, coupled with its degradation by glutaminase (98). However, the only evidence for this proposition is that glutamine synthetase activity is found in cultures that also have glutaminase activity (75, 98). Nevertheless, there is a reciprocal regulation between *E. coli* glutaminase B and glutamine synthetase. The former is inhibited by ATP and activated by AMP (75); the opposite applies to glutamine synthetase (108).

There are reports about the effect of a pulse of NH_4Cl on the levels of different metabolites in *E. coli* (84). Cultures growing in glucose-proline and pulsed with 10 mM NH_4Cl consumed 90% of their ATP in 20 s, and their glutamine pool increased 20-fold. At the same time, the glutamate concentration initially decreased and then gradually increased. GS activity rapidly decreased, because glutamine stimulates adenylylation. Afterwards, the glutamine concentration decreased as ATP increased (107). It is likely that the decrease in ATP, by releasing glutaminase B from partial inhibition, is responsible for the decrease in glutamine. It was proposed that deamidation of glutamine under those conditions made glutamate available for oxidation through the TCA cycle. This in turn resulted in an increase in ATP and, at the same time, reduced those biosynthetic, ATP-requiring processes which make use of the amide nitrogen of glutamine (76, 107). The energy expenditure in glutamine synthesis has also been documented for *S. typhimurium*; here, a mutant unable to adenylylate GS accumulates glutamine in excess and grows

poorly (46). The response of *N. crassa*, previously grown with limited ammonium in fed-batch or chemostat cultures, to the addition of an excess of nitrogen resembles the metabolic behavior described above. When an excess of ammonium was added to slowly growing, ammonium-limited cultures of *N. crassa*, the cultures began to grow exponentially only 4 h later (47). However, 1 h after the addition, the intracellular concentrations of ammonium and glutamine had risen 10- and 100-fold, respectively, and began to decrease only after 4 h. It is clear that the limitation of glutamine was not the reason for the absence of exponential growth immediately after the addition of ammonium. In fact, during this time, glutamine was synthesized and accumulated from carbon skeletons and energy accumulated during nitrogen limitation. Perhaps *N. crassa* does not grow immediately after the addition of ammonium because accumulated carbon and energy must be depleted and/or because the excess glutamine synthesis temporarily deprives the cells of energy. In any case, it appears that the cell is merely transforming accumulated energy from one chemical compound into another, a process reflected by suboptimal growth. What may be more important is that this displacement of energy from one form to another is an adaptation to variations in carbon and nitrogen supply. The energy accumulated in glutamine can be utilized very effectively afterwards, as shown by the rapid decrease of this amino acid after exponential growth begins following the upshift to high ammonium (R. A. Lomnitz, B.Sc. thesis, Universidad Nacional Autónoma de México, México City, 1983.).

We have presented information about the energy expenditure of glutamine synthesis and cycling in *N. crassa*. In addition, if the synthesis and cycling of glutamine are stopped, carbon flow from sucrose decreases drastically. It appears that energy must be expended to induce carbon flow and generate more energy. In this context, glutamine cycling is related to carbon utilization and regulation. It is significant that *Bacillus subtilis* glutamine synthesis mutants have an alteration in catabolite repression (24). *B. subtilis* GS mutants that lack almost all GS activity are partially released from glucose catabolite repression; the intracellular metabolite pool of these mutants suggests that this effect is the result of poor utilization of glucose (24).

Harder et al. (30) have proposed that biological energy conversion might have evolved to work under conditions of maximal energy output, with a consequent reduced efficiency in trapping the energy for biosynthetic processes. Tempest et al. (99) have stated that maximization of growth rate demands an optimization of the rates of flow of intermediates, reducing equivalents, and energy and that an optimization of energy flow requires the functioning of energy-dissipating reactions. The application of nonequilibrium thermodynamics to energy conversion in biological systems supports this proposition (33, 44, 92, 106). A simple model to regulate the transfer of energy from catabolism to anabolism is to interpose a cycle in which energy is dissipated in ATP-consuming reactions. When catabolism overloads the system with an excess of substrate, the extra carbon and energy can be used in cycling, as in the case of trehalose metabolism in *S. cerevisiae* in the presence of glucose at high temperatures (37). These processes are examples of a low-level coupling between the catabolic and anabolic process. However, under conditions when the carbon and energy generated by the catabolic process are restricted, the energy-dissipating systems operate at their minimum levels and an optimal coupling with anabolic process would be established. The continuous operation of a futile cycle is a necessary cost that must be paid by the cell to adjust rapidly to new conditions. In addition, the continuous use of energy by a futile cycle would be a driving reaction that sustains the system, generating energy more readily when needed than a system requiring activation. In conclusion, the cycling reactions would function as a buffer to stabilize variations in the system that produce and consume energy for cell activity (92).

An additional metabolic advantage can be envisaged for the operation of a glutamine cycle: it allocates, at a given time, a specific amount of carbon, energy, and nitrogen in the form of glutamine and/or glutamate to avoid inconvenient variations in nitrogen and carbon distribution. For instance, during nitrogen limitation, glutamate accumulates greatly and the glutamine concentration is very low (54, 63). The addition of an excess of nitrogen to nitrogen-deprived cultures results in the synthesis and accumulation of glutamine that can be used afterwards. Since most of the nitrogen is being cycled between glutamate and glutamine, it is to be expected that a substantial amount of carbon is sequestered. However, because carbon is also cycling, it can be delivered for catabolism according to the needs of the cell. The increased ability to regulate glutamine concentration during cycling may also be an advantage in regulating a compound that controls nitrogen metabolism. A consequence of the cycling of glutamine is that the energy expenditure necessary for its operation can also be a sensor of the energy available for the cell.

CONCLUSION

I postulate that the glutamine cycle is not futile, because it is required to drive an effective carbon flow to support growth and it facilitates the allocation of nitrogen and/or carbon according to cellular demands. It is an open question whether cycling of glutamine is unique to *N. crassa*. The presence of the glutamine cycle in other organisms is worth investigating.

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