UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO
Unidad Académica de los Ciclos Profesional
y de Posgrado del C.C.H.
Centro de Investigación sobre Fijación de Nitrógeno

La Síntesis y el Recambio de la Glutamina como Procesos Reguladores del Metabolismo del Nitrógeno y Carbono en Neurospora crassa.

T E S I S

Que para obtener el grado de
Doctora en Investigación Biomédica Básica
Presenta:

GEORGINA HERNANDEZ DELGADO

1988

CONTENIDO

1. INTRODUCCION Y ANTECEDENTES

1.1 EL METABOLISMO DEL NITROGENO

Rhizobium

Mucor rouxii

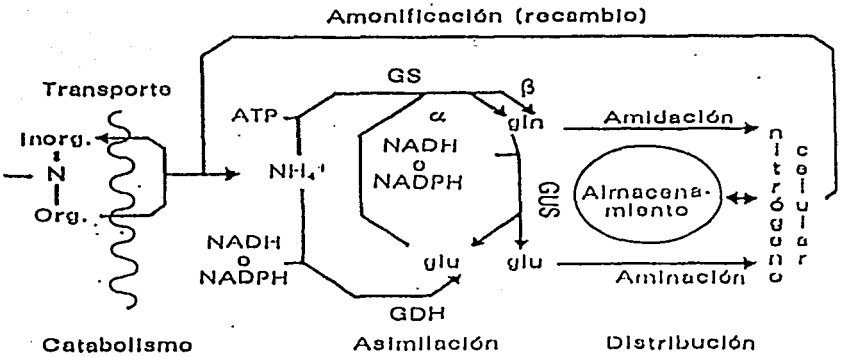


Fig. 1. Metabolismo del nitrógeno.

Tomada de: Mora, J., Hernández, G., y Lara, F. Metabolismo del Nitrógeno. En: "Bioquímica y Biología Molecular. Temas de Actualidad para Graduados." S. Ochoa, L. F. Leloir, J. Oró y A. Sols (eds). Salvat Editores, S. A., Barcelona, España, 1986.

Nota: En esta figura la glutamato sintasa (GOGAT) aparece como GUS.

Neurospora crassa

N. crassa

tiene un papel central en el metabolismo nitrogenado de algunos organismos. En los procariotas, como el cianobacterio heterocistoso no fijador *N. crassa*, la asimilación de amonio es esencial para el crecimiento, ya que no puede utilizar otras fuentes de nitrógeno.

1.2 LA ASIMILACION DE AMONIO

N. crassa

N. crassa

NADPH (GDH)

NADH (GOGAT)

GDH

GDH

N. crassa

GDH

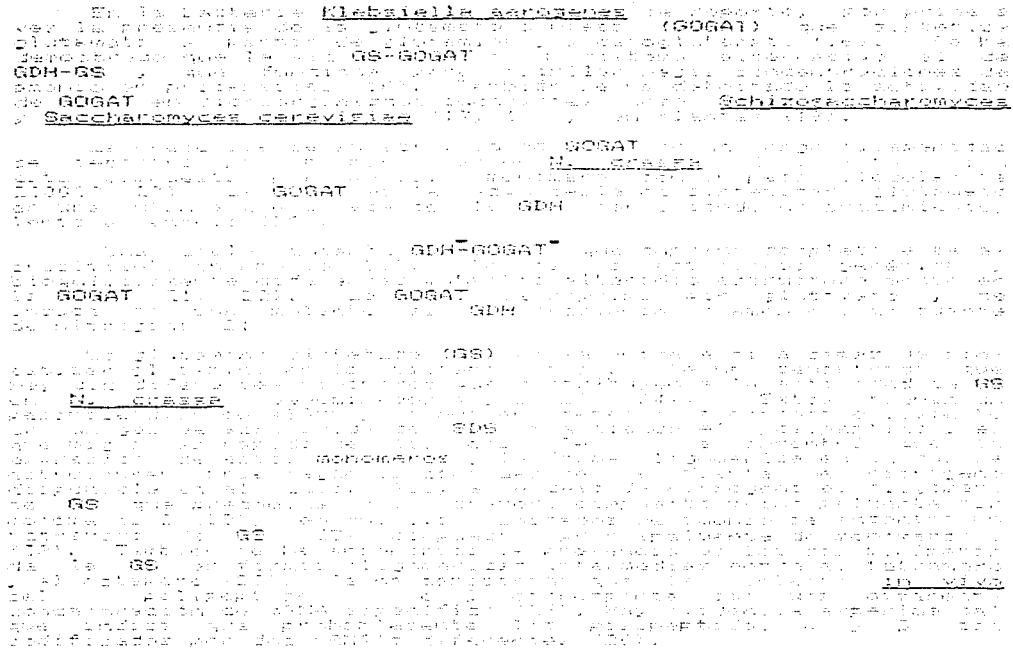
GDH

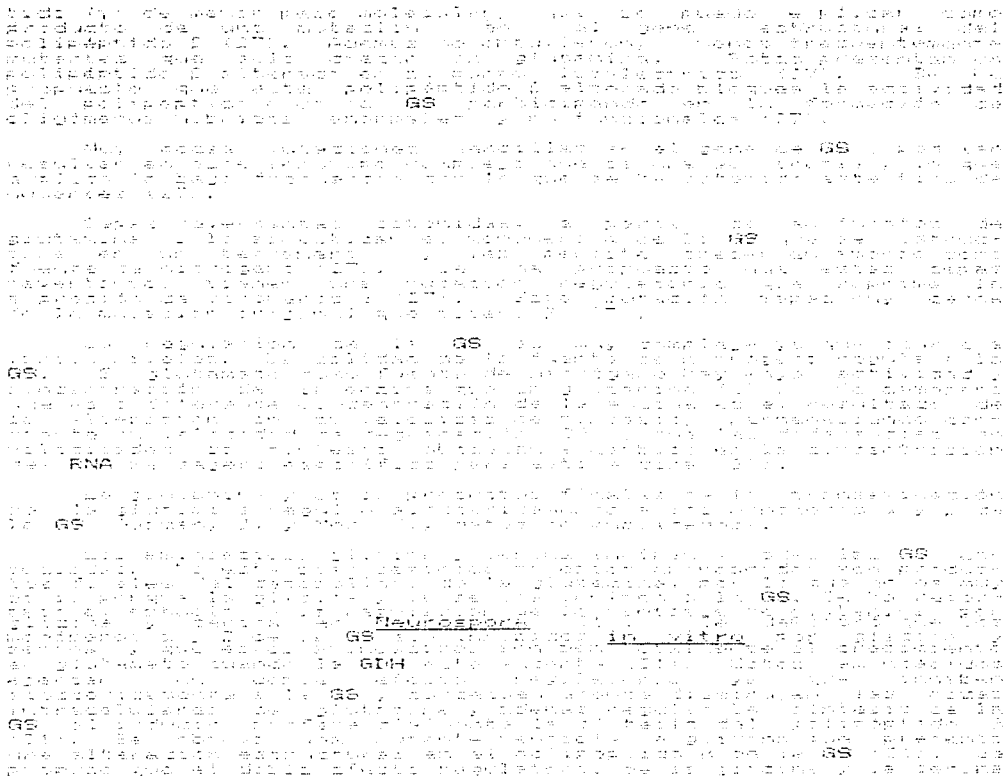
Neurospora

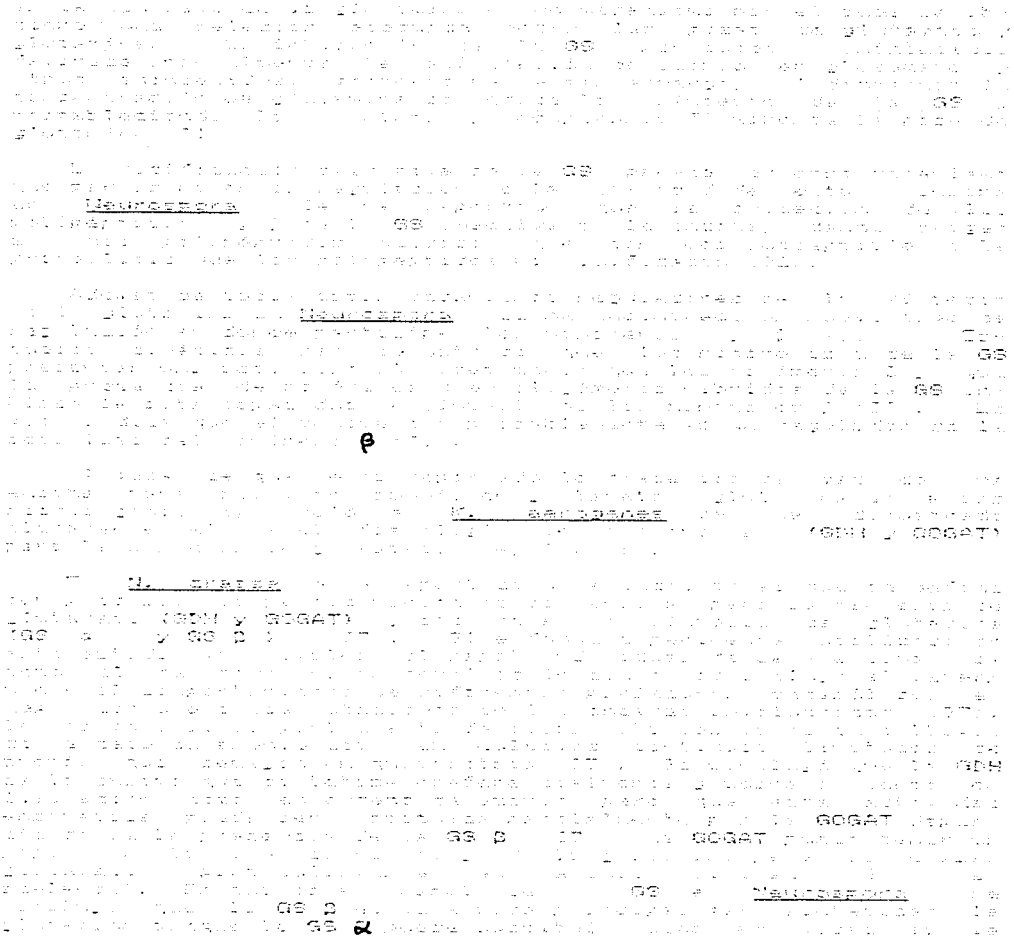
GDH

Neurospora

de novo


Klebsiella aerogenes
(GOGAT)
GS-GOGAT
GDH-GS
GOGAT
Schizosaccharomyces
Saccharomyces cerevisiae
GOGAT
N. crassa
GOGAT
GDH
GDH-GOGAT
GOGAT
GOGAT
GDH
(GS)
GS
N. crassa
GDH
monomers
GS
GS
GS
in vivo
GS
Neurospora
GS


GS
GS
GS
GS
RNA
GS
GS
GS
Neurospora
in vitro
GS
GDH
GS
GS
GS



EL RECAMBIO DE LA GLUTAMINA

N. crassa

N. crassa

N. crassa

GDH

Neurospora

Neurospora

Neurospora

Table

GDH

GDH

GS

GDH

GS

GDH

GOGAT

GOGAT

GOGAT

GOGAT

GS

GS

GDH [illegible] GOGAT [illegible]

[illegible] GOGAT [illegible]

GOGAT [illegible] GOGAT [illegible] GDH [illegible]

GOGAT [illegible]

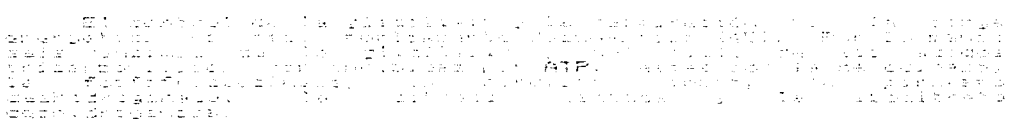
[illegible] N. crassa [illegible]

[illegible] N. crassa [illegible]

1.4 LA REGULACION COORDINADA DEL METABOLISMO DEL CARBONO Y DEL NITROGENO

[illegible]

[illegible] N. crassa



2.- OBJETIVOS Y METODO EXPERIMENTAL.

3. RESULTADOS

Glutamine Synthesis Regulates Sucrose Catabolism in *Neurospora crassa*

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The effect of glutamine synthesis on sucrose metabolism in *Neurospora crassa* was studied. Different inhibitors of glutamine synthetase were used to inhibit glutamine synthesis in mutants having a low ammonium assimilation capacity. Sucrose utilization was impaired, as indicated by a lower concentration and synthesis of intermediates of the tricarboxylic acid cycle and reduced release of CO_2. We propose that a coordinated regulation of carbon and nitrogen utilization is achieved through sensing of the carbon and nitrogen flows through glutamine synthesis, mediated by changes in the intracellular content of ATP, which is reduced as a consequence of glutamine synthesis.

INTRODUCTION

Carbon and nitrogen metabolism interact in the first instance in the reactions of ammonium assimilation. Carbon compounds, energy and reducing power are required for glutamate and glutamine synthesis. In *Neurospora crassa* glutamate can be synthesized through the participation of two different enzymes, glutamate dehydrogenase (GDH) (EC 1.4.1.4), NADPH dependent, which synthesizes glutamate from 2-oxogluturate and ammonium (Fincham, 1950; Hernández *et al.*, 1983) and glutamate synthase (GOGAT) (EC 1.4.1.14), NADH dependent, which gives two molecules of glutamate from glutamine and 2-oxoglutarate (Hummelt & Mora, 1980*a*, *b*). Glutamine synthetase (GS) (EC 6.3.1.2) requires ATP and in *N. crassa* it is found in two different oligomeric forms composed of α and β monomers respectively (Dávila *et al.*, 1980).

Carbon and nitrogen metabolism also interact in the metabolic steps related to the distribution of nitrogen from glutamate and glutamine into several biosynthetic pathways. In *N. crassa* the nitrogen from glutamine is distributed by the enzymes of the ω-amidase pathway as well as by transamidation. Glutamine transaminase synthesizes different amino acids and ω-amidase hydrolyses the resulting 2-oxoglutaramate to 2-oxoglutarate and ammonium (Calderón *et al.*, 1985). The ammonium released by the ω-amidase pathway is assimilated not only by GDH but also by GS, thus leading to the operation of a glutamine cycle in which this amino acid is continually degraded and resynthesized (Calderón *et al.*, 1985; Calderón & Mora, 1985). GOGAT also participates in the distribution of the nitrogen from glutamine (Calderón & Mora, 1985).

The regulation by the nitrogen source of the enzymes that synthesize glutamate or glutamine (Hernández *et al.*, 1983; Vichido *et al.*, 1978; Quinto *et al.*, 1977; Sánchez *et al.*, 1978) and the participation of these enzymes in different ammonium assimilation pathways in *N. crassa* (Lara *et al.*, 1982) have been reported. There have also been reports on the regulation of GS and of GDH by the carbon source in *N. crassa* and other micro-organisms (Ferguson & Sims, 1974*a*, *b*; Hemmings, 1978; Kapoor & Grover, 1970). Mora *et al.* (1980) reported that when mycelium of

Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MS, methionine sulphoximine.

N. crassa was deprived of the carbon source, the GS was degraded; they proposed that this could be a regulatory mechanism to conserve energy. However, despite the cost, in terms of carbon skeletons, reducing power and ATP, of the ammonium assimilation reactions, nothing is known about the effect of glutamine synthesis on carbon metabolism. We therefore looked for an effect of glutamine synthesis on sucrose metabolism in *N. crassa*.

METHODS

Strains. The *Neurospora crassa* wild-type strain *74A*, the GDH-deficient mutant *am-1* (GDH^-) and the GS partial auxotroph *gln-1a* (GS^s) were from the Fungal Genetics Stock Center (Humboldt State University Foundation, Arcata, Calif., USA). The double mutant *am-1;gln-1a* ($GDH^-;GS^s$) was from the collection of J. Mora.

Growth conditions. Batch cultures of *N. crassa* were grown at 37 °C on Vogel minimal medium (N) (Vogel, 1964) containing 1·5% (w/v) sucrose. At this growth temperature a higher GS activity allowed a higher growth rate in all the strains used and allowed growth of the glutamine auxotroph (GS^s) on glutamate (Dávila *et al.*, 1978). Other nitrogen sources used in place of or in addition to ammonium nitrate are stated in the text. Growth was measured as described previously (Lara *et al.*, 1982) by total protein concentration determined by the Lowry method.

Determination of GS activity. Cell-free extracts were prepared as previously described (Lara *et al.*, 1982; Vichido *et al.*, 1978). GS activity was measured as described by Ferguson & Sims (1974*a*). Specific activity was expressed as units (mg total protein)$^{-1}$, units being μmol γ-glutamyl hydroxamate produced min^{-1}.

Ammonium determination. Samples of the medium were collected and ammonium was measured with an Orion electrode (Espin *et al.*, 1979).

Determination of amino acid pools. Samples for amino acid analysis were prepared by homogenizing cells with 80% (v/v) ethanol (Hummelt & Mora, 1980*a*). The amino acids were separated in an Aminco amino acid analyser, coupled with orthophthalaldehyde (Sigma) and quantified in an Aminco Ratio Fluorometer.

Determination of other metabolites. Mycelial samples were collected on membrane filters (Millipore, type HA, 0·45 μm) and washed with distilled water. Glucose, 2-oxoglutarate and other organic acids were extracted by resuspending the samples in 0·6 M-$HClO_4$.

Glucose was determined with a glucose kit (Boehringer Mannheim). 2-Oxoglutarate was determined by measuring the initial rate of change in A_{340}, using bovine GDH. A sample of the extract was incubated at room temperature with 30 mM-ammonium sulphate, 0·1 mM-NADH and 0·01 ml of a 2·5% (v/v) solution of bovine liver GDH [50 units (mg protein)$^{-1}$] (Sigma product no. G2626) in 1 ml final volume of 100 mM-potassium phosphate buffer pH 7·6.

The intracellular concentration of pyruvate (Table 2) was determined by measuring the initial rate of change in A_{340}, using bovine lactic dehydrogenase (LDH). A sample of the extract was incubated at room temperature with 0·1 mM-NADH and 0·01 ml of a 2·5% (v/v) solution of LDH [400 units (mg protein)$^{-1}$] (Sigma product no. L1006) in 1 ml final volume of 100 mM-Tris buffer pH 7·8.

To measure the CO_2 released from [U-^{14}C]sucrose, samples of the culture were taken and transferred to a side-arm tube with fresh medium containing 0·06% sucrose. These cultures received a 15 min pulse with [U-^{14}C]sucrose (0·5 μCi ml^{-1}; 18·5 kBq ml^{-1}). The CO_2 released was collected with NaOH (Mora *et al.*, 1972). The same procedure was followed to determine the CO_2 released from [6-^{14}C]glucose; in this case the fresh medium contained 0·3% glucose and [6-^{14}C]glucose (0·5 μCi ml^{-1}; 18·5 kBq ml^{-1}).

Measurement of radioactivity incorporated into 2-oxoglutarate from [*U*-^{14}C]*sucrose.* Cultures were labelled for 15 min with [U-^{14}C]sucrose at 25 μCi ml^{-1} in 2 ml medium with the indicated nitrogen source and 0·06% sucrose as carbon source. 2-Oxoglutarate was extracted as described above, and was completely converted to glutamate using 0·1 ml of a 2·5% (v/v) solution of bovine liver GDH [50 units (mg protein)$^{-1}$] (Sigma; as above) and 60 mM-NADH in 100 mM-potassium phosphate buffer pH 7·6. The reaction was left for 40 min at room temperature and stopped with 80% (v/v) ethanol. The precipitated protein was separated by centrifugation and the supernatant, containing the amino acids and oxo acids, was lyophilized and resuspended in 150 μl lithium citrate buffer (0·30 M-Li^+, 0·053 M-citrate, pH 2·88) (Benson Co., Reno, Nev., USA; product no. 1216). The amino acids were seperated in an Aminco amino acid analyser and the fractions corresponding to glutamate were collected and counted in a liquid scintillation counter. Radioactivity specifically incorporated to 2-oxoglutarate was determined by subtracting the radioactivity incorporated into glutamate in a control without GDH from that incorporated when GDH was added.

Measurement of radioactivity incorporated into tricarboxylic acid cycle intermediates from [*U*-^{14}C]*sucrose.* Cultures were labelled for 15 min with [U-^{14}C]sucrose at 25 μCi ml^{-1} in 2 ml medium with the indicated nitrogen source and 0·06% sucrose as the carbon source. The mycelia were collected and the organic acids were extracted as described above. The extracts containing the organic acids were stored at 4 °C before separation and quantification of the tricarboxylic acid cycle intermediates by HPLC (J. Calderón and co-workers, unpublished). The fractions corresponding to the elution time of each tricarboxylic acid cycle intermediate were collected and their radioactivity was measured in a liquid scintillation counter.

Measurement of radioactivity incorporated into amino acids from [U-^{14}C]sucrose. Cultures were labelled for 15 min with [U-^{14}C]sucrose at 100 μCi ml^{-1} in 1 ml medium with the indicated nitrogen source and 0·06% sucrose as the carbon source. The mycelia were collected and the amino acid content was determined as described by Hummelt & Mora (1980*a*). The radioactivity incorporated into each amino acid was determined in a liquid scintillation counter.

Reproducibility of results. The experiments reported were each repeated at least once; representative results are shown.

RESULTS

Glutamine synthesis and carbon metabolism

The experimental approach used to study whether glutamine synthesis and carbon metabolism were coordinately regulated consisted in inhibiting glutamine synthesis and determining whether sucrose catabolism was also impaired. For this purpose we used mutant strains of *N. crassa* which had a low ammonium assimilation capacity, so as to make it easier to achieve a drastic inhibition of carbon and nitrogen flows.

The GDH$^-$ mutant strain has a residual growth on ammonium as nitrogen source (Fincham, 1950) due to the synthesis of glutamate by GOGAT (Hummelt & Mora, 1980*a*, *b*). In these circumstances both 2-oxoglutarate [32 nmol (mg protein)$^{-1}$] and ammonium (Fincham, 1950), substrates of GDH, accumulate intracellularly as a result of the partial block of glutamate synthesis. When glycine or serine is added to the medium, the growth of the GDH$^-$ mutant is totally arrested (Fincham, 1950). These amino acids are inhibitors of GS from several sources (Stadtman & Ginsburg, 1974). They are effective inhibitors of *N. crassa* GS activity *in vitro* at low glutamate concentrations and they also inhibit glutamine synthesis *in vivo* (Hernández *et al.*, 1986). We explain the complete blockage of growth on ammonium of the GDH$^-$ mutant by glycine and serine as the result of the inhibition of glutamine synthesis *in vivo*. We studied the relation between glutamine synthesis and carbon catabolism from sucrose in this GDH$^-$ mutant by inhibiting GS activity with glycine and serine and determining the intracellular pools of 2-oxoglutarate. After 12 h incubation under these conditions the intracellular pools of 2-oxoglutarate in the conidia were very low: 2·8 nmol (mg protein)$^{-1}$ on ammonium plus glycine and 2·4 nmol (mg protein)$^{-1}$ on ammonium plus serine. These results indicate an inhibition of sucrose catabolism which may also contribute to the growth inhibition of the GDH$^-$ mutant by glycine and serine. The inhibitory effect of these amino acids on growth of conidia of the GDH$^-$ mutant strain was reversible, since after 12 h incubation on ammonium plus an inhibitory amino acid the conidia were able to grow on ammonium as the sole nitrogen source (data not shown). To see whether the decreased 2-oxoglutarate content was specifically related to the inhibition of GS by glycine or serine, we tried to inhibit the growth of the GDH$^-$ mutant with methionine sulphoximine (MS), a specific inhibitor of GS activity (Ronzio & Meister, 1968). In this condition, the 2-oxoglutarate pool in the conidia was barely detectable [0·9 nmol (mg protein)$^{-1}$]. The same phenomenon was observed when growth of the wild-type strain on ammonium was inhibited with MS (data not shown). These results suggested that there could be a direct relation between glutamine synthesis and 2-oxoglutarate concentration.

The effect of the inhibition of glutamine synthesis on sucrose catabolism was directly tested by determining the synthesis of tricarboxylic acid cycle intermediates over a short period of time following transfer of previously grown mycelia of the GDH$^-$ mutant to ammonium or ammonium plus an inhibitor of GS (glycine or MS) (Table 1). At this time there was a very small difference in growth between each culture and the intracellular glucose content was similar in every condition (data not shown). The specific radioactivity of the tricarboxylic acid cycle intermediates, 2-oxoglutarate, pyruvate, succinate and malate, was lower in mycelium incubated on ammonium plus either glycine or MS; the effect was more apparent in the presence of glycine (Table 1).

These experiments indicate that the inhibition of glutamine synthesis prevents sucrose catabolism at the level of synthesis of the tricarboxylic acid cycle intermediates.

Table 1. *Synthesis of tricarboxylic acid cycle intermediates in vivo in the GDH⁻ mutant*

Cultures were grown for 12 h on glutamate and then transferred to the indicated nitrogen source. The concentrations used were 25 mM-ammonium, 50 mM-glycine and 3 mM-MS. The tricarboxylic acid cycle intermediates synthesized *in vivo* were quantified after 30 min incubation on the indicated nitrogen source; the cultures received a pulse with [U-^{14}C]sucrose as described in Methods. The experiment was repeated three times; representative results are shown.

Nitrogen source	Specific radioactivity (c.p.m. nmol^{-1})			
	2-Oxo-glutarate	Pyruvate	Succinate	Malate
Ammonium	706	252918	13016	419909
Ammonium + glycine	79	28598	3702	ND
Ammonium + MS	159	115978	10930	130423

ND, Not detected.

Table 2. *Metabolite contents in the GDH⁻;GS± mutant*

Conidia were incubated for 12 h with the indicated nitrogen sources. The amino acid concentrations used were 5 mM-glutamine and 50 mM-glycine, serine or leucine. The experiment was repeated twice; representative results are shown.

Nitrogen source	Growth (μg protein) ml^{-1})	Ammonium released [μmol (mg protein)$^{-1}$]	Metabolite content [nmol (mg protein)$^{-1}$]				
			Glutamate	Glutamine	2-Oxo-glutarate	Pyruvate	Glucose
Glutamine	321	6	136	ND	27	23	26
Glutamine + glycine	2	23	33	141	3	3	78
Glutamine + serine	0	16	7	422	5	3	85
Glutamine + leucine	77*	1	152	279	15	11	10

ND, Not detected.
*There was optimal growth, after an initial lag phase.

Glutamine recycling and carbon metabolism

Glutamine is a very good nitrogen source in *N. crassa* (Vichido *et al.*, 1978). However, mutants lacking some of the ammonium assimilation related enzyme activities, e.g. GDH⁻, GOGAT⁻;GDH⁻, and GDH⁻;GS± strains, have a slower growth rate and/or excrete ammonium into the medium when glutamine is used as nitrogen source (Calderón *et al.*, 1985; Calderón & Mora, 1985). This phenomenon is explained by the reduced capacity of the mutants to assimilate ammonium derived from glutamine degradation, thus indicating recycling of glutamine (Calderón & Mora, 1985).

Arrest of growth on ammonium when glutamine synthesis was inhibited was to be expected; however, as the inhibition of glutamine synthesis also affected carbon catabolism from sucrose (Table 1) this could be a sufficient condition for the inhibition of growth even with glutamine present. The latter assumption was investigated was investigated by using the double mutant GDH⁻;GS±, which has a low synthesis of both glutamate and glutamine, so that maximal inhibition of glutamine synthesis by glycine or serine could be achieved: these amino acids very effectively inhibit the *N. crassa* GS activity *in vitro* when glutamate concentration is low (Hernández *et al.*, 1986).

When glutamine was the sole nitrogen source, the culture grew optimally and excreted ammonium into the medium, and the glutamine pool was undetectable (Table 2). We interpret

Table 3. *Synthesis of tricarboxylic acid cycle intermediates in vivo and Co_2 release in the wild-type and GS⁻ mutant strains grown on glutamine*

Cultures were pregrown for 12 h on 5 mM-glutamate and then transferred to 5 mM-glutamine. The tricarboxylic acid cycle intermediates synthesized *in vivo* and CO_2 released from sucrose were quantified after 30 min incubation on glutamine; the cultures received a pulse with [U-^{14}C]sucrose as described in Methods. The experiment was repeated twice; representative results are shown.

Strain	Specific radioactivity (c.p.m. nmol^{-1}) 2-Oxoglutarate	Pyruvate	Succinate	Malate	CO_2 from sucrose [c.p.m. (mg protein)$^{-1}$]
Wild-type	1798	75075	19563	3527315	23520
GS⁻ mutant	96	16038	1813	69185	9137

the latter as being the result of the degradation of glutamine when the cells were grown on this amino acid as nitrogen source and of the very low capacity of the GDH⁻;GS⁻ mutant to reassimilate ammonia and to synthesize glutamine. In the presence of glutamine plus glycine or serine, the growth of the GDH⁻;GS⁻ mutant was completely abolished, and the non-growing conidia excreted more ammonium in proportion to their protein content than did the mycelium growing on glutamine alone (Table 2). Under these conditions, a decrease in intracellular glutamate and a large increase in intracellular glutamine were observed (Table 2). The increase in glutamine was probably due to accumulation from the medium after 12 h incubation in the absence of growth.

In this experiment leucine was used as a control because it competes with glutamine uptake in a similar way to the inhibitory amino acids (Pall, 1969) without inhibiting GS activity. In our conditions, glutamine uptake was lower in the presence of either glycine, serine or leucine than with glutamine alone (data not shown). When leucine was added to the medium together with glutamine, the GDH⁻;GS⁻ mutant was able to grow after a lag phase; its excretion of ammonium was barely detectable and it had high intracellular pools of glutamate and glutamine (Table 2).

When the GDH⁻;GS⁻ mutant was incubated on glutamine plus glycine or serine, the intracellular pools of pyruvate and 2-oxoglutarate in the inhibited conidia were very low as compared to those found in conidia germinating on glutamine alone or on glutamine plus leucine (Table 2). The intracellular pools of glucose were higher in the inhibited conidia incubated on glutamine plus glycine or serine than in those incubated on glutamine plus leucine or glutamine alone (Table 2). This indicates that the low pools of 2-oxoglutarate and pyruvate found on glutamine plus glycine or serine (Table 2) were not due to the lack of glucose. Another observation consistent with 2-oxoglutarate deficiency being responsible for the low glutamate content was the presence of a high activity of a glycine:2-oxoglutarate transaminase (data not shown), which could provide intracellular glutamate whenever glycine and 2-oxoglutarate were present.

The growth-inhibitory effect of glycine and serine on the GDH⁻;GS⁻ mutant strain incubated on glutamine was reversible, since the conidia were able to grow if the inhibitors were removed after 12 h.

We conclude that glutamine synthesis was required for growth even in the presence of this amino acid as the nitrogen source, since the inhibitory effect of glycine and serine on growth of the GDH⁻;GS⁻ mutant correlated with a deficiency of pyruvate, 2-oxoglutarate and glutamate, and not with glutamine limitation (Table 2).

The data presented in Table 2 lead us to propose that glutamine synthesis, rather than a particular absolute glutamine content, is necessary for optimal utilization of the carbon source, and that glutamine cycling is a necessary condition for growth even in the presence of glutamine. To obtain further support for this proposition, we measured sucrose catabolism in the presence of glutamine in the wild-type strain as compared to the GS⁻ mutant, which has a single mutation that alters the structure of the GSβ polypeptide (Dávila *et al.*, 1983), has a 20-fold lower GS

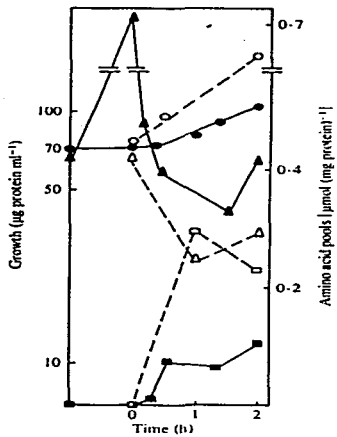

Fig. 1. Growth and amino acid pools of the GS⁺ mutant. The culture was grown for 12 h on 5 mM-glutamate and then the mycelium was washed and divided into two cultures. One culture (open symbols) was transferred to 5 mM-glutamine, at time 0. The other culture (filled symbols) was first incubated for 1 h in a medium without a nitrogen source and with 0·3 mM-MS, then washed and transferred to 5 mM-glutamine. ○, ●, Growth; △, ▲, intracellular glutamate; □, ■, intracellular glutamine. The experiment was repeated at least three times; representative results are shown.

activity than the wild-type and is able to grow on glutamate at 37 °C (Dávila *et al.*, 1978). The synthesis of tricarboxylic acid cycle intermediates *in vivo* and the CO_2 released from sucrose were measured 30 min after transfer of previously grown mycelia of the two strains from glutamate to glutamine as the nitrogen source, where their growth rate was similar. The specific radioactivity of 2-oxoglutarate, pyruvate, succinate and malate was higher in the wild-type than in the GS⁺ mutant strain when grown on glutamine (Table 3). In addition, the CO_2 released from [U-^{14}C]sucrose was lower in the GS⁺ mutant, whose synthesis of glutamine is lower. This effect was not due to a dilution of the label by glucose accumulation, since the total intracellular glucose content was 3·6-fold higher in the wild-type than in the GS⁺ mutant. The synthesis of glutamate was similar in both strains (data not shown). We propose that when glutamine synthesis is lower due to the partial block on GS activity in the GS⁺ mutant strain, carbon catabolism is impaired at the level of the synthesis of intermediates of the tricarboxylic acid cycle, reflected in a diminution of CO_2 released from sucrose (Table 3).

This proposal was further tested under conditions of maximal inhibition of GS activity. This was achieved by adding MS to the GS⁺ mutant, thus reducing the GS activity 150-fold as compared to that in the wild-type strain. Since MS is an irreversible inhibitor of GS (Ronzio & Meister, 1968), the GS activity was still inhibited 2 h after removal of the inhibitor (Table 4); activity was regained after 4 h as a result of enzyme synthesis (data not shown). The results of these experiments are shown in Fig. 1 and Table 4. In the presence of MS, no glutamine pool was detected and the glutamate pool increased (Fig. 1). When this culture was shifted to glutamine, it

Table 4. *GS activity, synthesis of glutamine, glutamate and 2-oxoglutarate in vivo and CO_2 release from glucose in the GS$^{\pm}$ strain*

After 12 h growth on 5 mM-glutamate the culture was washed and transferred to 5 mM-glutamine for 2 h (*a*) or to 0·5 mM-MS for 1 h followed by washing and transfer to 5 mM-glutamine for 2 h (*b*). All the parameters shown were measured after 2 h growth on glutamine. The experiment was repeated at least three times; representative results are shown.

Condition	GS specific activity [U (mg protein)$^{-1}$]	Glutamine from sucrose* (c.p.m. μmol^{-1})	Glutamate from sucrose* (c.p.m. μmol^{-1})	2-Oxoglutarate from sucrose* [c.p.m. (mg protein)$^{-1}$]	CO_2 from glucose† [c.p.m. (mg protein)$^{-1}$]
(*a*) Gln	0·0030	1225	5650	2124	4593
(*b*) MS→Gln	0·0004	163	3132	325	1652

* The cultures received a pulse of [U-^{14}C]sucrose.
† Different cultures received a pulse of [6-^{14}C]glucose.

had an initial lag phase that was followed by a slower growth despite its high glutamate content and sizeable glutamine pool (Fig. 1) as compared to the non-inhibited culture. Although the glutamine content was only 2-fold lower in the inhibited culture (Fig. 1) the glutamine synthesis varied 7·5-fold. We measured the carbon catabolism of sucrose after the shift to glutamine. The amount of CO_2 released from [6-^{14}C]glucose was lower when the GS was inhibited and a correlation with a decrease in 2-oxoglutarate synthesis from sucrose was also apparent (Table 4). This was supported by the lower glutamate synthesis observed when GS was inhibited (Table 4).

DISCUSSION

In this work we explored the relation between glutamine synthesis and carbon catabolism from sucrose. By using mutant strains (GDH$^-$, GS$^{\pm}$ and the double mutant GDH$^-$;GS$^{\pm}$) with a reduced ability to assimilate ammonium through glutamate, and glutamine and/or GS inhibitors, nitrogen and carbon metabolism were both blocked.

When mycelium of the GDH$^-$ mutant was incubated on ammonium plus an inhibitor of GS, sucrose catabolism decreased as shown by a lower synthesis of several tricarboxylic acid cycle intermediates (Table 1). This effect was observed after a short period of time during which growth was not affected. Since both glycine, which inhibits GS activity (Stadtman & Ginsburg, 1979; Hernández *et al.*, 1986), and MS, which is a specific GS inhibitor (Ronzio & Meister, 1968), gave the same effect (Table 1) we propose that the diminution in carbon catabolism was an effect specifically related to the inhibition of glutamine synthesis.

A relevant finding was that the inhibition of glutamine synthesis was sufficient to inhibit growth even in the presence of glutamine. We observed that with the GDH$^-$;GS$^{\pm}$ mutant on glutamine plus a GS inhibitory amino acid, growth was completely arrested and the intracellular levels of 2-oxoglutarate and pyruvate were low (Table 2). On glutamine plus leucine, not a GS inhibitor, there was a minor effect on the content of tricarboxylic acid cycle intermediates (Table 2). This can be explained by the lag phase that precedes growth under these conditions, and may also explain the glutamine accumulation (Table 2). In addition, the presence of the inhibitors was not essential since an impairment of sucrose catabolism was also observed in mycelia of a partial glutamine auxotroph (GS$^{\pm}$) growing on glutamine as the sole nitrogen source (Table 3).

The effect of inhibiting glutamine synthesis was further enhanced when MS, an inhibitor of GS activity, was added to the GS$^{\pm}$ mutant in the presence of glutamine (Table 4, Fig. 1). 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 and CO_2 release from [6-^{14}C]glucose (Table 4). The outcome was a low growth rate in the presence of a sizeable intracellular glutamine pool (Fig. 1).

These data thus support our conclusion that growth inhibition is not due to a lack of glutamine content. We have reported (Hernández *et al.*, 1986) that when growth and GS were inhibited by

glycine in the GS⁻ mutant in the presence of glutamate, the synthesis of glutamine *in vivo* was 100-fold lower while the absolute glutamine content was only 2-fold lower as compared to the culture growing on glutamate.

The data presented in this work indicate that in *N. crassa* there is coordinated regulation of carbon and nitrogen source utilization, i.e. when nitrogen assimilation was blocked through the inhibition of glutamine synthesis, carbon catabolism from sucrose decreased. This may explain the necessity for glutamine cycling (Calderón *et al.*, 1985; Calderón & Mora, 1985). The drain of ATP through glutamine synthesis and the regulation of carbon metabolism by energy charge could be considered as a point of interaction in the coordinated regulation of carbon and nitrogen metabolism. An observation consistent with this proposition was that in *N. crassa* the intracellular concentrations of ATP were raised when glutamine synthesis was inhibited (G. Hernández and co-workers, unpublished). It has been reported (Wohlhueter *et al.*, 1973) that when ammonium is added to an ammonium-limited culture of *Escherichia coli* there is a burst of glutamine synthesis which imposes a drastic drain on ATP; the accumulated glutamine activates the enzymes for GS inactivation by adenylylation and thus the ATP pool is replenished. These data suggest that the amount of ATP spent in glutamine synthesis may be important to the cell. The control of glycolysis and respiration by energy charge is well documented (Atkinson, 1977). We propose that whenever glutamine synthesis is blocked the energy status of the cell improves with the consequent inhibition of carbon catabolism. This coordinated regulatory mechanism could allow the cell to avoid the accumulation of carbon skeletons when synthesis of the universal nitrogen donors is blocked.

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REFERENCES

ATKINSON, D. (1977). *Cellular Energy Metabolism and its Regulation*. New York: Academic Press.

CALDERÓN, J. & MORA, J. (1985). Glutamine cycling in *Neurospora crassa*. *Journal of General Microbiology* **131**, 3237–3242.

CALDERÓN, J., MORETT, E. & MORA, J. (1985). The ω-amidase pathway in the degradation of glutamine in *Neurospora crassa*. *Journal of Bacteriology* **161**, 807–809.

DÁVILA, G., SÁNCHEZ, F., PALACIOS R. & MORA, J. (1978). Genetics and physiology of *Neurospora crassa* glutamine auxotrophs. *Journal of Bacteriology* **134**, 693–698.

DÁVILA, G., LARA, M., GUZMÁN, J. & MORA, J. (1980). Relation between structure and function of *Neurospora crassa* glutamine synthetase. *Biochemical and Biophysical Research Communications* **92**, 134–140.

DÁVILA, G., BROM, S., MORA, Y., PALACIOS, R. & MORA, J. (1983). Genetic and biochemical characterization of glutamine synthetase from *Neurospora crassa* glutamine auxotrophs and their revertants. *Journal of Bacteriology* **156**, 993–1000.

ESPÍN, G., PALACIOS, R. & MORA, J. (1979). Glutamine metabolism in nitrogen-starved conidia of *Neurospora crassa*. *Journal of General Microbiology* **115**, 59–68.

FERGUSON A. R. & SIMS, A. P. (1974*a*). The regulation of glutamine metabolism in *Candida utilis*: the role of glutamine in the control of glutamine synthetase. *Journal of General Microbiology* **80**, 159–171.

FERGUSON, A. R. & SIMS, A. P. (1974*b*). The regulation of glutamine metabolism in *Candida utilis*: the inactivation of glutamine synthetase. *Journal of General Microbiology* **80**, 173–185.

FINCHAM, J. R. S. (1950). Mutant strains of *Neurospora* deficient in aminating ability. *Journal of Biological Chemistry* **181**, 61–73.

HEMMINGS, B. A. (1978). Evidence for the degradation of nicotinamide adenine dinucleotide phosphate-dependent glutamate dehydrogenase of *Candida utilis* during rapid enzyme inactivation. *Journal of Bacteriology* **133**, 867–877.

HERNÁNDEZ, G., SÁNCHEZ-PESCADOR, R., PALACIOS, R. & MORA, J. (1983). Nitrogen source regulates glutamate dehydrogenase NADP synthesis in *Neurospora crassa*. *Journal of Bacteriology* **154**, 524–528.

HERNÁNDEZ, G., MORA, Y. & MORA, J. (1986). Regulation of glutamine synthesis by glycine and serine in *Neurospora crassa*. *Journal of Bacteriology* **165**, 133–138.

HUMMELT, G. & MORA, J. (1980*a*). NADH-dependent glutamate synthase and nitrogen metabolism in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **92**, 127–133.

HUMMELT, G. & MORA, J. (1980*b*). Regulation and function of glutamate synthase in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **96**, 1688–1694.

KAPOOR, M. & GROVER, A. K. (1970). Catabolite-controlled regulation of glutamate dehydrogenases of *Neurospora crassa*. *Canadian Journal of Microbiology* **16**, 33–40.

LARA, M., BLANCO, L., CAMPOMANES, M., CALVA, E., PALACIOS, R. & MORA, J. (1982). Physiology of

ammonium assimilation in *Neurospora crassa*. *Journal of Bacteriology* **150**, 105–112.

MORA, J., SALCEDA, R. & SÁNCHEZ, S. (1972). Regulation of arginase activity by intermediates of the arginine biosynthetic pathway in *Neurospora crassa*. *Journal of Bacteriology* **110**, 870–877.

MORA, Y., CHÁVEZ, O. & MORA, J. (1980). Regulation of *Neurospora crassa* glutamine synthetase by carbon and nitrogen source. *Journal of General Microbiology* **118**, 455–463.

PALL, M. L. (1969). Amino acid transport in *Neurospora crassa*. I. Properties of two amino acid transport systems. *Biochimica et biophysica acta* **203**, 139–149.

QUINTO, C., MORA, J. & PALACIOS, R. (1977). *Neurospora crassa* glutamine synthetase. Role of enzyme synthesis and degradation on the regulation of enzyme concentration during exponential growth. *Journal of Biological Chemistry* **252**, 8724–8727.

RONZIO, R. & MEISTER, A. (1968). Phosphorylation of methionine sulfoximine by glutamine synthetase. *Proceedings of the National Academy of Sciences of the United States of America* **59**, 164–170.

SÁNCHEZ, F., CAMPOMANES, M., QUINTO, C., HANSBERG, W., MORA, J. & PALACIOS, R. (1978). Nitrogen source regulates glutamine synthetase mRNA levels in *Neurospora crassa*. *Journal of Bacteriology* **136**, 880–885.

STADTMAN, E. R. & GINSBURG, A. (1974). The glutamine synthetase of *Escherichia coli*: structure and control. In *The Enzymes*, vol. 10, pp. 755–807. Edited by P. D. Boyer. New York: Academic Press.

VICHIDO, I., MORA, Y., QUINTO, C., PALACIOS, R. & MORA, J. (1978). Nitrogen regulation of glutamine synthetase in *Neurospora crassa*. *Journal of General Microbiology* **106**, 251–259.

VOGEL, H. J. (1964). Distribution of lysine pathways among fungi: evolutionary implications. *American Naturalist* **98**, 435–446.

WOHLHUETER, R. M., SCHUTT, M. & HOLZER, H. (1973). Regulation of glutamine synthesis *in vivo* in *Escherichia coli*. In *The Enzymes of Glutamine Metabolism*, pp. 45–64. Edited by S. Prusiner & E. R. Stadtman. New York: Academic Press.

Regulation of Carbon and Nitrogen Flow by Glutamate Synthase in *Neurospora crassa*

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A glycine-resistant *Neurospora crassa* mutant (*am-132 ; gly*^{r}), derived from the *am-132* mutant, was isolated and characterized. [*am-132* itself has a deletion in the structural gene for NADP-dependent glutamate dehydrogenase (GDH).] This new mutation also conferred resistance to serine and methionine sulphoximine (MS), which are inhibitors of glutamine synthetase (GS). In addition, the mutant obtained grew better on ammonium than the *am-132* parental strain. Resistance to glycine was not due to increased synthesis of glutamine by an altered or induced GS, nor to increased glutamate synthesis by induction of the catabolic NAD-dependent GDH, nor to NADH-dependent glutamate synthase (GOGAT), which was as sensitive to inhibitors as the GOGAT from the parental strain. The glycine-resistance mutation lowered but did not abolish the carbon flow; this resulted in a lower content of tricarboxylic acid cycle intermediates. GOGAT activity was inhibited *in vitro* by several organic acids and methionine sulphone (MSF). The higher growth rate of the glycine-resistant mutant on ammonium or on ammonium plus glycine, serine or MS was explained by an increased capacity of GOGAT to synthesize glutamate *in vivo* due to a lower content of inhibitory tricarboxylic acid cycle intermediates; the higher glutamate content overcomes the effect of the GS inhibitors and explains the MSF resistance of the mutant.

INTRODUCTION

The regulation of nitrogen metabolism in *Neurospora crassa* has been investigated previously (Hummelt & Mora, 1980*a*, *b*; Hernández *et al.*, 1983; Lara *et al.*, 1982; Vichido *et al.*, 1978). The growth on ammonium of a *N. crassa* mutant that lacks NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4) is inhibited by the amino acids glycine and serine (Fincham, 1950). This is due to the inhibition of glutamine synthetase (GS) (EC 6.3.1.2) which results in the diminution of the carbon flow (Hernández, *et al.*, 1986). When nitrogen assimilation is blocked by inhibition of glutamine synthesis, carbon catabolism from sucrose decreases (Hernández & Mora, 1986). We proposed that the drain of ATP through glutamine synthesis and the regulation of carbon metabolism by the energy charge could be considered as a point of interaction in the coordinated regulation of carbon and nitrogen metabolism.

On this basis, we now report the isolation and characterization of a glycine-resistant (*gly*^{r}) mutant strain of *N. crassa*, that has altered carbon and nitrogen flows.

METHODS

Strain. The *Neurospora crassa* wild-type strain 74A was from the Fungal Genetics Stock Center (Humboldt State University Foundation, Arcata, Calif., USA). The mutant strain *am-132a*, which has a deletion in the NADP-dependent GDH structural gene (Kinsey & Hung, 1981), was kindly provided by J. A. Kinsey, Department of Microbiology, University of Kansas Medical Center, Kansas 66103, USA.

Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MS, methionine sulphoximine; MSF, methionine sulphone.

Growth conditions. Batch cultures of *N. crassa* were grown at 37 °C on Vogel's minimal medium (N) (Vogel, 1964) containing 1·5% (w/v) sucrose. Other nitrogen sources used in addition to ammonium nitrate are stated in the text. Growth was measured as total protein (determined by the Lówry method) as described previously (Lara *et al.*, 1982; Vichido *et al.*, 1978).

Mutagenesis and mutant selection. A conidial suspension of strain *am-132a* was prepared and adjusted to 4×10^6 conidia ml^{-1} in sterile water. A sample of the suspension (5 ml) was placed in a Petri dish and exposed to direct UV radiation for 60 s. After the mutagenic treatment, 0·2 ml of the suspension were plated on N agar medium (Vogel, 1964) supplemented with glucose and fructose (0·05%, w/v, each), 2% (w/v) sorbose and 25 mM-glycine. Plates were incubated in the dark at 29 °C until colonies appeared (2 to 7 d), and then transferred to slants of N agar medium supplemented with 1·5% (w/v) sucrose, and 25 mM-glycine. Crosses were made in Corn meal agar (34 $g\ l^{-1}$) (Difco). Spot testing and progeny analysis were done as described by Davis & De Serres (1970).

Determination of enzyme activities. This was done in cell-free extracts of *N. crassa* prepared as described previously (Lara *et al.*, 1982; Vichido *et al.*, 1978). GS activity was measured as described by Ferguson & Sims (1974). NAD-dependent GDH (EC 1.4.1.2) was measured as described by Fincham (1957). NADH-linked glutamate synthase (GOGAT) (EC 1.4.1.14) was measured by following NADH oxidation as described by Hummelt & Mora (1980*a*). Enzyme activities are expressed as units (mg protein)$^{-1}$. One unit is the amount of activity that transforms 1 μmol substrate min^{-1}.

In the experiments where GOGAT activity was inhibited *in vitro*, the enzyme was purified as described by Hummelt & Mora (1980*b*), except that the DEAE column was equilibrated with 0·05 M-phosphate buffer. A 14-fold purified preparation was obtained. The final concentration of the organic acids was 10 mM and the solutions were adjusted to pH 8·6 with 1 M-KOH.

Determination of amino acids. Samples for amino acid analysis were prepared by homogenizing cells with 80% (v/v) ethanol (Hummelt & Mora, 1980*a*). The amino acids were separated by using an Aminco amino acid analyser, and after being coupled with orthophthalaldehyde (Sigma) were quantified in an Aminco ratio fluorimeter.

Determination of tricarboxylic acid cycle intermediates. Mycelial or conidial samples were collected on membrane filters (Millipore type HA, 0·45 μm) and washed with distilled water. Organic acids were extracted by resuspending the samples in 0·6 M-$HClO_4$ and neutralizing with 2·0 M-K_2PO_4; they were then determined in the clear supernatant after centrifugation. The extracts were stored at 4 °C before the determinations were done. 2-Oxoglutarate was determined by measuring the initial rate of change in A_{340} using bovine GDH. A sample of the extract was incubated at room temperature with 30 mM-ammonium sulphate, 0·1 mM-NADH and 0·01 ml 2·5% (v/v) bovine liver GDH [50 units (mg protein)$^{-1}$] (Sigma no. G2626) in 1 ml final volume 100 mM-potassium phosphate buffer, pH 7·6.

The other tricarboxylic acid cycle intermediates were separated and quantified by HPLC [method of J. Calderón (unpublished)].

Reproducibility of results. The experiments reported were each repeated at least once; representative results are shown.

RESULTS

Genetic and phenotypic analysis

The *am-132* mutant has a deletion in the NADP-dependent GDH structural gene (GDH$^-$) (Kinsey & Hung, 1981) and is unable to grow on ammonium plus glycine (Fig. 1) or serine, like other mutant strains which have a point mutation in the structural gene of this enzyme (Fincham, 1950; Brett *et al.*, 1976). The *am-132* mutant was used as the parental strain for mutant selection. One of the *am-132* glycine-resistant (*gly^r*) mutants isolated was crossed with the wild-type strain 74A. One *am-132 ;gly^r* mutant obtained from this cross was backcrossed again with the wild-type strain and the progeny were analysed: 57% were wild-type, 24% *am-132* and 19% *am-132 ;gly^r*. These results indicated that the *gly^r* mutation segregated as a monogenic mutation. The work we report here was done with a GDH$^-$ *am-132 ;gly^r* double mutant obtained from the latter cross.

Fig. 1 shows the growth of the *am-132 ;gly^r* mutant on ammonium plus glycine compared to the *am-132* single mutant, which is unable to grow on this nitrogen source (Fincham, 1950), and the wild-type strain, which had a higher growth rate. Unexpectedly, the growth rate of the *am-132 ;gly^r* mutant on ammonium was higher than that of the GDH$^-$ strain (Fig. 1), but lower than that of the wild-type strain. This same phenomenon was observed when the cultures were grown at 25 °C (data not shown), when the growth rate on ammonium of the GDH$^-$ mutant strain (*am*) is considerably lower (Hummelt & Mora, 1980*b*) than at 37 °C (Fig. 1). In addition, this new

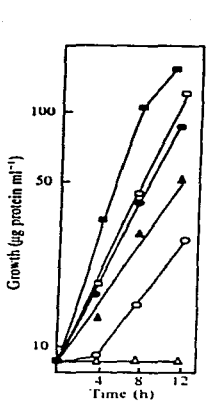

Fig. 1

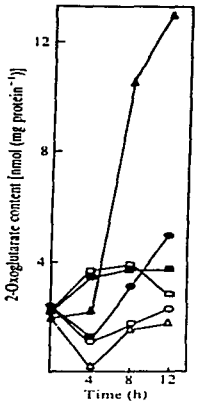

Fig. 2

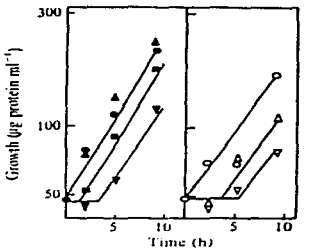

Fig. 3

Fig. 1. Growth of the *am-132 ;gly*^{r} (●, ○) and *am-132* (▲, △) mutants and the wild-type strain (■, □) of *N. crassa* on ammonium (●, ▲, ■) or ammonium + 50 mM-glycine (○, △, □). The experiment was done four times; results from a representative experiment are shown.

Fig. 2. Intracellular content of 2-oxoglutarate of the *am-132 ;gly*^{r} (●, ○) and *am-132* (▲, △) mutants and the wild-type strain (■, □) of *N. crassa* grown on ammonium (●, ▲, ■) or ammonium + 50 mM-glycine (○, △, □). The experiment was done three times; representative results are shown.

Fig. 3. Inhibition *in vivo* of growth of the *am-132 ;gly*^{r} and *am-132* mutants by MSF. Conidia were pregerminated for 10 h in minimal medium at 37 °C with agitation; MSF was then added to each culture. ●, ■, ▲, ▼, *am-132 ;gly*^{r} mutant; ○, △, ▽, *am-132* mutant. ●, ○, Minimal medium without MSF; △, ▲, 0·01 mM-MSF; ■, 0·05 mM-MSF; ▼, ▽, 0·1 mM-MSF. The experiment was done twice; representative results are shown.

mutation also conferred resistance to serine and to methionine sulphoximine (MS) (data not shown), a specific GS inhibitor (Ronzio & Meister, 1968).

Activities of ammonium-assimilation enzymes in the am-132 ;gly^{r} *mutant*

We reported previously the isolation of a glycine-sensitive mutant that maps in the structural gene of GS and has an altered and inactive β polypeptide in its GS (Hernández *et al.*, 1986). However, the *am-132 ;gly*^{r} mutant has a wild-type GS as indicated by the M_r and isoelectric point of its α and β GS monomers (data not shown). As mentioned before, the *gly*^{r} mutation segregated independently from the *am-132* mutation, which indicates that this new mutation did not map in the structural gene of GS since the *am* and *gln* loci both map in the VR chromosome and are closely linked (Sober, 1970).

GS activity was not considerably increased in the *am-132 ;gly*^{r} mutant compared to the *am-132* strain, either when grown on ammonium or on ammonium plus glycine (Table 1). High GS activity was not responsible for growth on glycine as the *am-132* mutant had higher GS activity (Table 1) and was unable to grow on ammonium plus glycine (Fig. 1). The *in vitro* activity of the GS from the *am-132 ;gly*^{r} mutant was inhibited by glycine in a similar manner to the GS from the *am-132* mutant (data not shown).

Table 1. *Enzyme activities in the am-132 and am-132 ;gly^r mutants*

Enzyme activities (units) were determined after 12 h incubation on the nitrogen sources indicated. The experiment was done twice; representative results are shown.

Strain	Nitrogen source	GS*	GOGAT	NAD-dependent GDH
am-132	Ammonium	0·029	0·008	0·001
	Ammonium + 50 mM-glycine	0·116	0·004	0·001
am-132 ;gly^r	Ammonium	0·032	0·010	0·002
	Ammonium + 50 mM-glycine	0·052	0·009	0·001

* GS activity was determined by the synthetase assay.

Table 2. *Inhibition of GOGAT activity in vitro by MSF in the am-132 ;gly^r and am-132 mutants*

The experiment was repeated twice; representative results are shown.

MSF concn (mM)	Percentage inhibition	
	am-132	*am-132 ;gly^r*
0·05	60	67
0·20	74	80
0·40	100	100

The residual growth of the *am-132* mutant or other *am* mutants on ammonium is due to the presence of GOGAT (Hummelt & Mora, 1980*a*, *b*). It has also been shown that GOGAT does not completely replace NADP-dependent GDH in cultures with an excess of ammonium as the nitrogen source. (Hummelt & Mora, 1980*a*, *b*).

The GOGAT activity of the *am-132 ;gly^r* mutant was similar to that of the *am-132* mutant (Table 1). So, glycine-resistance cannot be explained by higher induction of GOGAT thus compensating for GS inhibition by glycine, which depends on glutamate concentration (Hernández *et al.*, 1986).

Methionine sulphone (MSF) is a very potent inhibitor of GOGAT from *Saccharomyces cerevisiae* (Masters & Meister, 1982). We found that 0·4 mM-MSF completely inhibited GOGAT activity in a dialysed cell extract of the *am-132* mutant grown on ammonium (Table 2). GOGAT from the *am-132 ;gly^r* mutant was also inhibited *in vitro* by MSF (Table 2), which suggests that the glycine-resistance mutation does not result in a structural alteration of GOGAT.

A *Saccharomyces cerevisiae* mutant that lacks biosynthetic NADP-dependent GDH activity was reported to regain its ability to grow optimally on ammonium when its catabolic NAD-dependent GDH was induced to very high levels (Grenson *et al.*, 1974). This was not the case for the *N. crassa* glycine-resistant mutant since the catabolic NAD-dependent GDH activity was very low in the *am-132 ;gly^r* mutant as well as in the *am-132* strain (Table 1).

Metabolic analysis of the am-132 ;gly^r mutant

If the *gly^r* mutation could have an effect on the utilization and/or flow of the carbon source, this could result in increased glutamate synthesis. The GDH$^-$ mutant did not accumulate 2-oxoglutarate when incubated with ammonium plus glycine (Fig. 2) and the 2-oxoglutarate pools of the *am-132 ;gly^r* double mutant grown on this nitrogen source were slightly but significantly higher (Fig. 2). In the *am-132* mutant the pyruvate and succinate contents were higher on ammonium than on ammonium plus glycine as the nitrogen source (Table 3). In the *am-132 ;gly^r* double mutant the intracellular pools of pyruvate and succinate were similar in the two growth conditions, and they were lower than in the *am-132* single mutant (Table 3).

Table 3. *Metabolite contents of the am-132 and am-132 ;gly^r mutants*

Metabolite contents [μmol (mg protein)$^{-1}$] were determined after 12 h incubation on the nitrogen sources indicated. The experiment was done twice; representative results are shown. ND, Not detected.

Strain	Nitrogen source	Pyruvate	Succinate	Glycine	Glutamate	Glutamine	Alanine
am-132	Ammonium	0·377	1·338	0·036	0·092	0·235	0·154
	Ammonium + 50 mM-glycine	0·219	0·881	2·570	ND	0·029	0·001
am-132;gly^r	Ammonium	0·157	0·593	0·056	0·057	0·185	0·093
	Ammonium + 50 mM-glycine	0·134	0·632	1·015	0·005	0·112	0·007

We conclude that the *am-132* mutant accumulated several tricarboxylic acid cycle intermediates due to the blockage in glutamate synthesis by NADP-dependent GDH; in the presence of glycine this accumulation did not occur (Fig. 2; Table 3), which may indicate that the carbon flow stops in the latter condition. In the *am-132;gly^r* double mutant grown on ammonium, there was a low content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3), which suggests a decrease in carbon flow; when glycine was added the lower content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3) suggested that the carbon flow was only slightly decreased as compared to the former condition. The contents of 2-oxoglutarate (Fig. 2), and pyruvate and succinate (Table 3), in the *am-132;gly^r* double mutant were closely similar to those of the wild-type strain (Fig. 2). The glycine-resistance mutation was not related to the transport of glycine into the cell since glycine ws accumulated by the *am-132;gly^r* mutant grown on ammonium plus glycine (Table 3).

A correlation between the content of tricarboxylic acid cycle intermediates and amino acids was observed as expected. The *am-132* mutant contained no detectable glutamate and had a very low alanine and glutamate content on ammonium plus glycine (Table 3) where its growth was abolished (Fig. 1), while the *am-132;gly^r* double mutant had a detectable glutamate pool (Table 3) and its content of alanine and glutamine increased several-fold when grown thus.

From these data we conclude that the *am-132;gly^r* mutant synthesizes more glutamate, alanine and glutamine, which is related to its higher capacity to grow on ammonium with or without glycine, and perhaps to a reduced carbon flow.

Curiously, the amino acid pools were higher in the *am-132* mutant than in the *am-132;gly^r* double mutant when grown on ammonium alone (Table 3). However, the data shown in Table 3 represent the total metabolic pools accumulated after 12 h incubation on the media indicated, and not the nitrogen and carbon flows of this mutant strain, a result which is discussed below.

Regulation of GOGAT activity by organic acids

From the experimental data shown, it appears that due to the blockage in the *am-132* mutant the intracellular content of several tricarboxylic acid cycle intermediates increases, and this, in turn, lowers the capacity of this mutant to synthesize glutamate. We tested if the latter effect could be due to the inhibition of GOGAT by organic acids. We determined the percentage inhibition of GOGAT activity in a partial purified enzyme preparation of the *am-132* mutant grown on ammonium in the presence of different concentrations of several organic acids that are tricarboxylic acid cycle intermediates, or the oxoacids of glycine or serine (Table 4). Glycine did not inhibit *N. crassa* GOGAT activity *in vitro* (data not shown).

GOGAT from the *am-132;gly^r* mutant was inhibited by organic acids in a similar manner to the GOGAT from the *am-132* mutant (data not shown). This supports our proposition that the glycine-resistance mutation is not due to a structural mutation in GOGAT, which could give a more resistant enzyme. Glycine resistance could rather be related to an increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132;gly^r* mutant due to a lower concentration of tricarboxylic acid cycle intermediates (Fig. 2; Table 3) that are inhibitors of GOGAT activity (Table 4).

Table 3. *Metabolite contents of the am-132 and am-132;gly^r mutants*

Metabolite contents [μmol (mg protein)$^{-1}$] were determined after 12 h incubation on the nitrogen sources indicated. The experiment was done twice; representative results are shown. ND, Not detected.

Strain	Nitrogen source	Pyruvate	Succinate	Glycine	Glutamate	Glutamine	Alanine
am-132	Ammonium	0·377	1·338	0·036	0·092	0·235	0·154
	Ammonium + 50 mM-glycine	0·219	0·881	2·570	ND	0·029	0·001
am-132;gly^r	Ammonium	0·157	0·593	0·056	0·057	0·185	0·093
	Ammonium + 50 mM-glycine	0·134	0·632	1·015	0·005	0·112	0·007

We conclude that the *am-132* mutant accumulated several tricarboxylic acid cycle intermediates due to the blockage in glutamate synthesis by NADP-dependent GDH; in the presence of glycine this accumulation did not occur (Fig. 2; Table 3), which may indicate that the carbon flow stops in the latter condition. In the *am-132;gly^r* double mutant grown on ammonium, there was a low content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3), which suggests a decrease in carbon flow; when glycine was added the lower content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3) suggested that the carbon flow was only slightly decreased as compared to the former condition. The contents of 2-oxoglutarate (Fig. 2), and pyruvate and succinate (Table 3), in the *am-132;gly^r* double mutant were closely similar to those of the wild-type strain (Fig. 2). The glycine-resistance mutation was not related to the transport of glycine into the cell since glycine ws accumulated by the *am-132;gly^r* mutant grown on ammonium plus glycine (Table 3).

A correlation between the content of tricarboxylic acid cycle intermediates and amino acids was observed as expected. The *am-132* mutant contained no detectable glutamate and had a very low alanine and glutamate content on ammonium plus glycine (Table 3) where its growth was abolished (Fig. 1), while the *am-132;gly^r* double mutant had a detectable glutamate pool (Table 3) and its content of alanine and glutamine increased several-fold when grown thus.

From these data we conclude that the *am-132;gly^r* mutant synthesizes more glutamate, alanine and glutamine, which is related to its higher capacity to grow on ammonium with or without glycine, and perhaps to a reduced carbon flow.

Curiously, the amino acid pools were higher in the *am-132* mutant than in the *am-132;gly^r* double mutant when grown on ammonium alone (Table 3). However, the data shown in Table 3 represent the total metabolic pools accumulated after 12 h incubation on the media indicated, and not the nitrogen and carbon flows of this mutant strain, a result which is discussed below.

Regulation of GOGAT activity by organic acids

From the experimental data shown, it appears that due to the blockage in the *am-132* mutant the intracellular content of several tricarboxylic acid cycle intermediates increases, and this, in turn, lowers the capacity of this mutant to synthesize glutamate. We tested if the latter effect could be due to the inhibition of GOGAT by organic acids. We determined the percentage inhibition of GOGAT activity in a partial purified enzyme preparation of the *am-132* mutant grown on ammonium in the presence of different concentrations of several organic acids that are tricarboxylic acid cycle intermediates, or the oxoacids of glycine or serine (Table 4). Glycine did not inhibit *N. crassa* GOGAT activity *in vitro* (data not shown).

GOGAT from the *am-132;gly^r* mutant was inhibited by organic acids in a similar manner to the GOGAT from the *am-132* mutant (data not shown). This supports our proposition that the glycine-resistance mutation is not due to a structural mutation in GOGAT, which could give a more resistant enzyme. Glycine resistance could rather be related to an increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132;gly^r* mutant due to a lower concentration of tricarboxylic acid cycle intermediates (Fig. 2; Table 3) that are inhibitors of GOGAT activity (Table 4).

Table 4. *Inhibition of GOGAT activity by organic acids in the am-132 mutant*

Mycelium was grown for 24 h on minimal medium; GOGAT was partially purified as described in Methods. The concentration of organic acids was 10 mM; the pH was adjusted to 8·6. The experiment was done twice; representative results are shown.

Organic acid	2-Oxoglutarate concn . . .	Percentage inhibition 0·05 mM	0·1 mM
None		0	0
Succinate		25	19
Malate		67	40
Citrate		25	20
Isocitrate		40	20
Fumarate		51	31
Pyruvate		25	19
Glyoxalate		40	19
β-Hydroxypyruvate		36	31

This postulated increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132 ; gly*^{r} mutant was investigated by determining its capacity to grow on ammonium plus MSF, compared with the *am-132* single mutant. Pregerminated conidia of the *am-132 ; gly*^{r} mutant had a higher growth rate on ammonium plus 0·1 mM-MSF than pregerminated conidia of the *am-132* mutant under the same conditions (Fig. 3). A higher growth rate on ammonium plus MSF was observed in the *am-132 ; gly*^{r} mutant, which has lower concentration of tricarboxylic acid cycle intermediates than the *am-132* mutant.

We thus conclude that in the *am-132 ; gly*^{r} mutant there is higher GOGAT activity *in vivo* than there is in the *am-132* mutant, due to the lower concentration in the double-mutant of organic acids (Fig. 2; Table 3) that are GOGAT inhibitors (Table 4). As a result of this increased capacity to synthesize glutamate (Table 3) the glycine-resistant mutant is able to grow better than the parental strain on ammonium plus a GS inhibitor such as glycine (Fig. 1), serine or MS and on ammonium plus the GOGAT inhibitor MSF (Table 2; Fig. 3).

DISCUSSION

Fincham (1950) reported that the amino acids glycine or serine completely inhibit growth of *am* strains on ammonium. We have demonstrated that the effect of glycine and serine is due to inhibition of GS activity (Hernández *et al.*, 1986). It proved possible to obtain a glycine-sensitive strain from wild-type *N. crassa* by a mutation that inactivates the highly active β polypeptide of GS (Hernández *et al.*, 1986).

When growth on glutamate of a partial glutamine auxotroph was inhibited by glycine, it was reported that synthesis of glutamate *in vivo* decreased 100-fold, even though the content of glutamine only decreased by half; this suggested that the synthesis of glutamine rather than the absolute content of this amino acid, played an important role in optimum cell growth (Hernández *et al.*, 1986). As a result of GS inhibition by glycine, or MS, the carbon flow decreases, and thus growth is prevented due to the double effect of lowering glutamine synthesis and carbon flow (Hernández & Mora, 1986).

In this paper we reported the isolation of a glycine-resistant mutant from the GDH$^-$ strain *am-132* that has a deletion in the structural gene for NADP-dependent GDH (Kinsey & Hung, 1981). This mutation appears to result in diminished carbon flow that cannot be decreased further by the presence of glycine; in the *am-132 ; gly*^{r} double mutant this results in a lower content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3). This is different from what occurs in the GDH$^-$ strain *am-132*, where the carbon flow was almost abolished and GS was totally inhibited in the presence of glycine, as shown by the decrease in glutamate, glutamine, alanine, pyruvate, succinate (Table 3) and 2-oxoglutarate contents (Fig. 2). Although in the

am-132 strain incubated with ammonium plus glycine, the 2-oxoglutarate content was more drastically reduced (Fig. 2) as compared to the pyruvate and succinate contents (Table 3), even these values represent reduced levels, since they were obtained after 12 h incubation in the absence of growth.

GOGAT from *N. crassa* was inhibited *in vitro* by different tricarboxylic acid cycle intermediates and also by the oxoacids of glycine and serine (Table 4). It has been reported that tricarboxylic acid cycle intermediates also regulate GOGAT from *E. coli* (Miller & Stadman, 1972). West *et al.* (1967) and Ashby *et al.* (1974) reported that *N. crassa* NADP-dependent GDH is activated cooperatively by several non-substrate tricarboxylic acid cycle intermediates.

The principal function of GOGAT could be related to the interconversion of glutamine to glutamate and to glutamate recycling (Calderón & Mora, 1985). This proposal is supported by the fact that GOGAT cannot completely replace NADP-dependent GDH in a GDH$^-$ mutant strain that has a decreased growth rate on ammonium (Fig. 1), where a low carbon flow and an accumulation of carbon skeletons is observed (Fig. 2; Table 3).

Taking into account that some organic acids were effective inhibitors of GOGAT activity (Table 4), a lower content of these tricarboxylic acid cycle intermediates in the *am-132 ;gly^r* mutant would allow a higher glutamate content (Table 3). This increased glutamate content could overcome GS inhibition by glycine, which depends on glutamate concentration (Hernández *et al.*, 1986). Another observation that supports the increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132 ;gly^r* mutants is that this strain had a higher growth rate on ammonium plus MSF, a GOGAT inhibitor, than the *am-132* mutant (Fig. 3).

The ability of the *am-132 ;gly^r* mutant to grow in the presence of glycine, serine or MS added individually to ammonium as the nitrogen source indicates that resistance is the result of a common effect of glycine, serine and MS. These three GS inhibitors had similar effects on glutamine synthesis and on sucrose catabolism, as shown here and by Hernández & Mora (1986).

We propose that in the *am-132 ;gly^r* mutant an increased carbon flow compared to that of the *am-132* mutant elicits increased glutamate and glutamine synthesis that is responsible for an increased growth rate on ammonium (Fig. 1). This proposal can also explain the capacity of the *am-132 ;gly^r* mutant to grow in the presence of glycine, serine, MS or MSF.

By the same token, the slow growth of the *am-132* mutant on ammonium as the sole nitrogen source (Fig. 1) is due to the accumulation of tricarboxylic acid cycle intermediates (Fig. 2; Table 3); this accumulation results in an inhibition of GOGAT that limits glutamine synthesis, which then reduces the carbon flow (Table 3). This proposal is supported by the accumulation of glutamate and other amino acids (Table 3), observed in the *am-132* mutant after 12 h slow growth on ammonium (Fig. 1); we assume that this limited growth is due to a reduced carbon flow. During the first hours of growth on ammonium, glutamate and glutamine pools were lower in the *am-132* mutant than in the *am-132 ;gly^r* mutant (data not shown).

It can be speculated that a glycolytic enzyme less sensitive to ATP inhibition may explain the *gly^r* phenotype.

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REFERENCES

Ashby, B., Wootton, J. C. & Fincham, J. R. S. (1974). *Biochemical Journal* **143**, 317–329.

Brett, M., Chambers, G. K., Holder, A. A., Fincham, J. R. S. & Wootton, J. C. (1976). Mutational amino acid replacements in *Neurospora crassa* NADP-specific glutamate dehydrogenase. *Journal of Molecular Biology* **106**, 1–22.

Calderón, J. & Mora, J. (1985). Glutamine cycling in *Neurospora crassa*. *Journal of General Microbiology* **131**, 3237–3242.

Davis, R. H. & De Serres, F. J. (1970). Genetic and microbiological research techniques for *Neurospora crassa*. *Methods in Enzymology* **17**, 79–143.

Ferguson, A. R. & Sims, A. P. (1974). The regulation of glutamine metabolism in *Candida utilis*: the role of glutamine in the control of glutamine synthetase. *Journal of General Microbiology* **80**, 159–171.

Fincham, J. R. S. (1950). Mutant strain of *Neurospora* deficient in aminating ability. *Journal of Biological Chemistry* **181**, 61–73.

FINCHAM, J. R. S. (1957). A modified glutamic acid dehydrogenase as a result of gene mutation in *Neurospora crassa*. *Biochemical Journal* **65**, 721–723.

GRENSON, M., DUBOIS, E., PIOTROWSKA, M., DRILLIEN, R. & AIGLE, M. (1974). Ammonium assimilation in *Saccharomyces cerevisiae* as mediated by two glutamate dehydrogenases. *Molecular and General Genetics* **128**, 73–85.

HERNÁNDEZ, G. & MORA, J. (1986). Glutamine synthesis regulates sucrose catabolism in *Neurospora crassa*. *Journal of General Microbiology* **132**, 3315–3323.

HERNÁNDEZ, G., SÁNCHEZ-PESCADOR, R., PALACIOS, R. & MORA, J. (1983). Nitrogen source regulates glutamate-dehydrogenase NADP synthesis in *Neurospora crassa*. *Journal of Bacteriology* **154**, 524–528.

HERNÁNDEZ, G., MORA, Y & MORA, J. (1986). Regulation of glutamine synthesis by glycine and serine in *Neurospora crassa*. *Journal of Bacteriology* **165**, 133–138.

HUMMELT, G. & MORA, J. (1980*a*). NADH-dependent glutamate synthase and nitrogen metabolism in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **92**, 127–133.

HUMMELT, G. & MORA, J. (1980*b*). Regulation and function of glutamate synthase in *Neurospora crassa*. *Biochemical and Biophysical Research Communications* **96**, 1688–1694.

KINSEY, J. A. & HUNG, B. S. T. (1981). Mutation at the *am* locus of *Neurospora crassa*. *Genetics* **99**, 405–414.

LARA, M., BLANCO, L., CAMPOMANES, M., CALVA, E., PALACIOS, R. & MORA, J. (1982). Physiology of ammonium assimilation in *Neurospora crassa*. *Journal of Bacteriology* **150**, 105–112.

MASTERS, D. S., JR & MEISTER, A. (1982). Inhibition by homocysteine sulfonamide of glutamate synthase purified from *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **257**, 8711–8715.

MILLER, R. E. & STADMAN, E. R. (1972). Glutamate synthase from *Escherichia coli*: an iron–sulfide flavoprotein. *Journal of Biological Chemistry* **247**, 7407–7419.

RONZIO, R. & MEISTER, A. (1968). Phosphorylation of methionine sulfoximine by glutamine synthetase. *Proceedings of the National Academy of Sciences of the United States of America* **59**, 164–170.

SOBER, H. A. (1970). (ed.). Selected data for molecular biology. In *Handbook of Biochemistry*, pp. 168–69. Cleveland, Ohio: Chemical Rubber Co.

VICHIDO, I., MORA, Y., QUINTO, C., PALACIOS, R. & MORA, J. (1978). Nitrogen regulation of glutamine synthetase in *Neurospora crassa*. *Journal of General Microbiology* **106**, 251–259.

VOGEL, H. J. (1964). Distribution of lysine pathways among fungi: evolutionary implications. *American Naturalist* **98**, 435–446.

WEST, D. J., TUVESON, R. W., BARRAT, R. W. & FINCHAM, J. R. S. (1967). Allosteric effects in nicotamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from *Neurospora crassa*. *Journal of Biological Chemistry* **242**, 2134–2138.

DISCUSION

N. crassa

ATP

ATP

ATP

GDH

GS

ATP

ATP

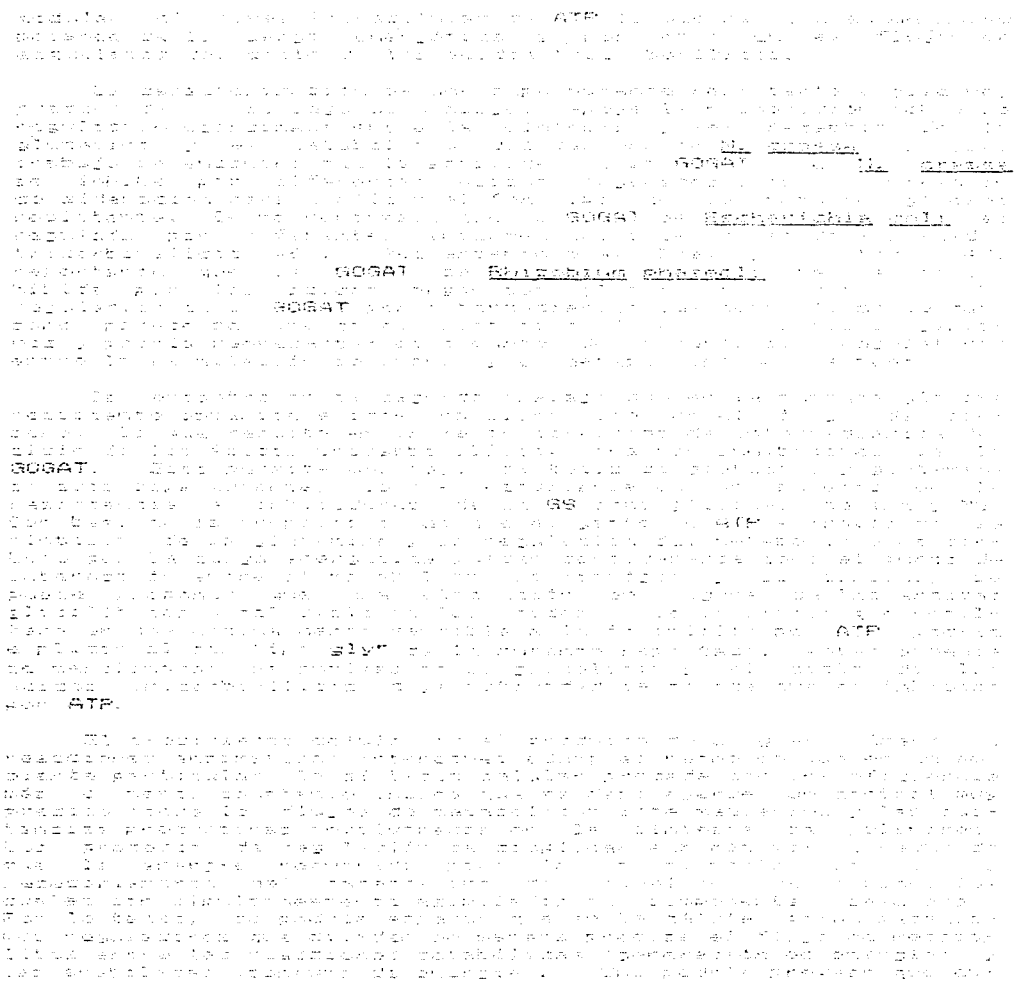
TABLA 1

Table 8
ATP CONTENT OF THE *GDH*⁻ MUTANT STRAIN

Nitrogen source	ATP (nmol/mgp)
Ammonium	0.62
Ammonium + glycine	8.44
Ammonium + MS	11.33

Note: Cultures were grown for 12 hr on glutamate as nitrogen source and transferred to the indicated medium for 30 min.

Tomada de: Mora, J., Calderón, J., and Hernández, G. Search, Assimilation and Turnover of Nitrogen in some Fungi. In: "Nitrogen Source Control of Microbial Processes". S. Sánchez-Esquivel (ed). CRC Press, Inc. 1988. In press.



ATP

E. coli

K. coli

ATP

GS

ATP

ATP

Penicillin

subtilis

B. subtilis

B.

subtilis

B. subtilis