FISIOLOGIA DE LA ASIMILACION DE AMONIO EN EL HONGO NEUROSPORA crassa.

T E S I S
PARA OBTENER EL GRADO DE MAESTRO EN
INVESTIGACION BIOMEDICA BASICA.

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CENTRO DE INVESTIGACION SOBRE FIJACION
DE NITROGENO.

UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO.

A Lulu mi esposa.

Por su amor su bondad y ternura.

A mis padres y hermanos.

Por su cariño su estimulo y apoyo.

A Jaime Mora.
Por su amistad su enseñanza
y paciencia.

A Rafael Palacios.
Por su amistad y su apoyo.

A mis compañeros.
Muchas gracias.

INTRODUCCION.

Este trabajo forma parte del proyecto de investigación que sobre el metabolismo nitrogenado en el hongo Neurospora crassa se ha desarrollado desde hace algunos años en los laboratorios de los Doctores Jaime Mora y Rafael Palacios, en el Instituto de Investigaciones Biomédicas y actualmente en el Centro de Investigación sobre Fijación de Nitrógeno. Este proyecto se ha enfocado principalmente hacia la síntesis de glutamina, la cual se lleva a cabo a través de la glutamino sintetasa (GS). Esta enzima juega un papel fundamental en el metabolismo nitrogenado de la célula, ya que el producto de la reacción que cataliza (la glutamina), es el principal donador de nitrógeno celular. Con el fin de que el trabajo aquí presentado se ubique en un contexto general del metabolismo nitrogenado en la célula, se revisarán brevemente:

a) Las diferentes vías de asimilación de amonio en diversos organismos y en Neurospora crassa.
b) El papel de la glutamina como donador de nitrógeno celular.
c) Las características de la reacción catalizada por la glutamino sintetasa.
d) Las características de la glutamino sintetasa en diversos organismos.
e) Las características de la glutamino sintetasa en Neurospora.

Vías de asimilación de amonio.

Procariotes:

Por mucho tiempo se consideró que a través de la glutamato deshidrogenasa (GDH) y la GS la célula incorporaba nitrógeno en compuestos orgánicos conforme a las ecuaciones I y II.

$$\alpha\text{-cetoglutarato} + NH_4^+ + NADPH \xleftrightarrow{GDH} \text{ac. glutámico.} \quad (I)$$

$$\text{ac. glutámico} + ATP + NH_4^+ \xleftrightarrow{GS} \text{glutamina.} \quad (II)$$

No fué sino hasta 1970 cuando Tempest v col. en experimentos con Klebsiella aerogenes encontraron en cultivos limitados de amonio que la actividad de la GDH era menor que en cultivos no limitados y que un minuto después de agregar 10mM de amonio a cultivos limitados de este compuesto, la poza de glutamina aumentaba 25 veces; por otro lado la alta Km de la GDH y la baja de GS para el amonio, los llevó a proponer que la GS es la primera enzima de asimilación de amonio y a describir la actividad de una enzima que transfiere el grupo amido de la glutamina al α-cetoglutarato para la síntesis de glutámico. Por ello la vía de asimilación de amonio a bajas concentraciones, en procariotes, es a través de las enzimas GS y la glutamato sintasa (GOGAT), como se muestra en las reacciones III y IV.

$$\text{ac. glutámico} + NH_4^+ + ATP \xleftrightarrow{GS} \text{glutamina.} \quad (III)$$

$$\text{glutamina} + \alpha\text{-cetoglutarato} + NADPH \xleftrightarrow{GOGAT} 2 \text{ glutámico.} \quad (IV)$$

Levaduras y Hongos.

Las enzimas GDH y GS han sido estudiadas ampliamente en hongos. La GDH existe como dos proteínas diferentes, una dependiente de NADH y la otra de NADPH; ambas tienen una afinidad baja por amonio (Km 10-50mM), pero se considera que la enzima dependiente de NADPH es la responsable de asimilar el nitrógeno.

Por otro lado se han hecho esfuerzos para determinar la presencia de GOGAT en estos organismos. Roon y col., (1974) han purificado la GOGAT de Saccharomyces cerevisiae, pero su función en la asimilación de nitrógeno es dudosa, ya que se encuentra en muy baja concentración. Algunos miembros del género Schizosaccharomyces, presentan una actividad de GOGAT, que permite asegurar que la via GS-GOGAT es la responsable de la asimilación de nitrógeno en este organismo (Brown et al., 1972).

Los estudios con ^{15}N. de Folkes y Sims (1974) en C. utilis demuestran que el 75% de la marca se incorpora inicialmente en glutámico y el 12-15% se incorpora posteriormente en glutamina, lo que demuestra que en C. utilis, la asimilación de nitrógeno es a través de la GDH.

Plantas.

La distribución de las enzimas que participan en la asimilación de nitrógeno en los diferentes organelos celulares de la planta, ha permitido definir las vías de asimilación de nitrógeno en las diferentes partes de la célula. En esta forma general, se ha demostrado la presencia de actividad de GS tanto en citosol como en cloroplastos de hojas o plástidos de raíz. El 80% de la actividad de GDH se localiza en mitocondrias, mientras que el 80% de la activi

dad de GOGAT está presente en cloroplastos (Wallsgrove et al., 1979).

Hojas.

Experimentos de incorporación de ^{13}N, realizados en hojas de espinacas, tanto en la luz como en la obscuridad, muestran que la marca se incorpora inicialmente en el grupo amido de la glutamina y posteriormente en el amino del glutámico; esta incorporación se bloquea al agregar metionina-sulfoximina (MS) que inhibe la actividad de GS (Ito et al., 1978).

Raíces.

Experimentos en raíz de arroz muestran que el tratamiento con MS inhibe en un 90% la incorporación de ^{15}N a glutamina y un 77% a glutámico (Arima y Kurazawa 1977). Por otro lado, Lewis (1977) ha encontrado que el ^{15}N se incorpora inicialmente a la glutamina y posteriormente al glutámico. En cultivos de tejido, estudios con ^{15}N demuestran que la marca se localiza en la glutamina en los primeros 10 segundos y que sólo después de 30 segundos la marca se encuentra en el glutámico (Skokut et al., 1978), además la adición de azaserina, inhibe la incorporación y síntesis de glutámico en un 99%.

Todos los datos anteriores, establecen que en plantas, la vía mas importante de asimilación de nitrógeno es a través de las enzimas GS y GOGAT.

Vías de asimilación de amonio en Neurospora.

Al igual que otros organismos, Neurospora presenta una actividad de GDH dependiente de NADPH, que cataliza la síntesis de ácido glutámico a partir de -cetoglutarato y amonio. Aún cuando la afinidad de esta enzima por el amonio es baja, por mucho tiempo se pensó que la asimilación de nitrógeno en Neurospora era a través de la GDH. Por otro lado, la actividad específica de esta enzima es regulada por la fuente de nitrógeno, siendo 5 veces mayor la actividad en exceso de amonio que en glutamina como fuente de nitrógeno. Esta diferencia corresponde a cambios en el nivel de síntesis de la GDH (Hernández et al., en preparación).

Estudios recientes en cultivos limitados de amonio, muestran que la cepa am-I de Neurospora que carece de la GDH biosintética, crece igual que la cepa silvestre en estas condiciones, esto es debido a la presencia de la glutamato sintasa (GOGAT) en este hongo, (Hummelt et al., 1980).

La actividad de GOGAT en Neurospora establece que la asimilación de amonio se lleva a cabo no sólo a través de la GDH y la GS sino también a través de la vía GS-GOGAT.

Las condiciones en las cuales participa una u otra vía, y su relación con las características de la GS serán expuestas posteriormente.

La glutamina como donador del nitrógeno celular. -

La glutamina juega un papel central en el metabolismo nitrogenado de la célula, ya que además de ser un aminoácido que se requiere para la síntesis de proteínas, es la fuente de nitrógeno más importante en diversas vías biosintéticas.

Por un lado el grupo α -amino de la glutamina, participa en la síntesis de diversos aminoácidos, esta transaminación de glutamina con α-cetoácidos se lleva a cabo por una transaminasa específica de glutamina, que da como productos de la reacción aminoácidos y α-cetoglutaramato el cual por la - actividad de una omega amidasa, se hidroliza produciendo amOnio y α-cetoglutarato. Estas reacciones forman parte del ciclo de la glutamina propuesto por Meister (Meister 1962).

Por otro lado el nitrógeno amido de la glutamina permite la síntesis de compuestos como purinas pirimidinas, coenzimas, aminoazúcares, y algunos aminoácidos; por ejemplo, se sabe que el nitrógeno del grupo indol del triptofano y un nitrógeno del imidazol de la histidina derivan del amido de la glutamina. Por otro lado, el grupo amido de la glutamina, se usa para la síntesis de carbamil fosfato, que es precursor tanto de primidinas como de arginina.

Las enzimas que catalizan la utilización del nitrógeno amido de la glutamina se denominan amidotransferasas de glutamina, y estan compuestas en su mayoría por 2 subunidades, donde la de menor peso molecular presenta una actividad de glutaminasa y son

inhibidas irreversiblemente por compuestos como 6-diazo-5-oxonorleucina o 2-amino-4 oxo-5 cloropentanoato.

Una de estas enzimas, recientemente descrita (Tempest et al., 1970), la glutamato sintasa (GOGAT), que cataliza la aminación del α-cetoglutarato a partir de glutamina, para la síntesis de 2 moléculas de ácido glutámico apoya aún más el papel central de la glutamina en la distribución del nitrógeno celular para la biosíntesis de aminoácidos y otros compuestos.

Características de la reacción catalizada por la Glutamino Sintetasa.

Por ser la glutamina el principal donador de nitrógeno en la célula, la glutamino sintetasa (GS), que es la enzima que catali za la biosíntesis de este aminoácido, a partir de amonio y ác. glutámico, juega un papel central en el metabolísmo nitrogenado de la célula. Esta enzima está presente en todo el reino animal y ha sido caracterizada en diferentes organismos (Bacterias, algas, hongos, plantas superiores y mamíferos).

La glutamino sintetasa cataliza la síntesis de glutamina como se muestra en la siguiente reacción.

Ac. glutámico + ATP + NH_4^+= glutamina + ADP + Pi

Además del NH_4^+, puede utilizarse hidroxilamina como subtra to de la glutamino sintetasa, dando como resultado la formación de γ-glutamilhidroxamato. Esta reacción se lleva a cabo en dos formas: una la llamada reacción de sintetasa a partir de glutámico y ATP (ecuación I) y otra la reacción de transferasa a partir de glutamina y ADP (ecuación II)

I: ATP + Ac. glutámico + NH_2OH = γ-glutamilhidroxamato + ADP + Pi

II: Glutamina + NH_2OH + ADP + Pi = γ-glutamilhidroxamato + NH_3^+

Características de la glutamino sintetasa en diferentes organísmos.

Estructura de la GS en procariotes:

La glutamino sintetasa de E. coli y Bacilluos subtilis, tiene un peso molecular de 590,000 a 600,000 y consta de 12 subunidades idénticas, cada una con un peso molecular de 50,000

(Meister 1974, Stadtman and Ginsburg 1974); las subunidades forman anillos hexagonales, dos de los cuales forman los do decámeros característicos de la enzima nativa.

En cianobacterias como Anabaena cylindrica, la glutamino sintetasa tiene un peso molecular entre 550,000 y 660,000 y está compuesta por 12 subunidades de un peso molecular de 49,000, las cuales se estructuran de manera similar a las de E. coli.

Estructura de la GS en eucariotes:

La glutamino sintetasa de Neurospora crassa está formada por subunidades que tienen un peso molecular de 48,000, estructurados como octámeros. Las fotografías de la enzima pura en el microscopio electrónico muestran anillos de cuatro subunidades, dos de las cuales conforman el octámero de la enzima. (Palacios 1976). En Candida utilis esta enzima se estructura en un octámero con un peso molecular de 390,000 y una conformación similar a la de Neurospora.

En las plantas, la glutamino sintetasa tiene un peso molecular que varía entre 350,000 y 400,000; al igual que las enzimas antes descritas, la GS de plantas consta de 8 subunidades. Al microscopio electrónico el octámero de GS del nódulo del frijol de soya se compone de 2 tetrámeros planares (McParlan 1976).

Diversas isozimas de la glutamino sintetasa.

Recientemente se han identificado dos formas de la glutamino sintetasa en Rhizobium japonicum (Darrow y Knotts 1977), y en Bacillus caldoliticus (Wedler et al., 1978), en ambos casos las dos isozimas de glutamino sintetasa difieren en su punto isoeléctrico y en su estabilidad.

En Rhizobium japonicum la GS tipo I es parecida a la de E. coli en su estructura, y por ser susceptible de adenilación, mientras que la GS tipo II no se adenila.

En plantas se han encontrado por cromatografía de intercambio iónico dos formas diferentes de GS en frijol de soya (Stasiewicz and Durman 1979). En hojas de cebada (Mann et al., 1979) y en arroz (Guiz et al., 1979). Las dos formas de GS en cebada, denominadas GS-I y GS-II, tienen un peso molecular similar, pero su estabilidad y su pH óptimo son diferentes. Estudios de localización en hojas, raíces y semillas, sugieren que la GS-I se encuentra en estos 3 órganos de la planta; sin embargo, la GS-II se localiza solamente en tejidos verdes (Mann et al., 1980); además experimentos en cebada muestran que la GS-I está en el citosol y que la GS-II se localiza en los cloroplastos.

Características de la glutamino sintetasa de Neurospora crassa.

La glutamino sintetasa de Neurospora es regulada tanto por la fuente de nitrógeno, como por la fuente de carbón. (Mora, Y., et al., 1980, Vichido et al., 1978). Cuando la cepa silvestre 74-A de Neurospora se crece en glutámico como fuente de nitrógeno, la actividad específica de GS es 10 veces mayor que en glutamina (Vichido et al., 1978), esta diferencia en la actividad de GS se debe a una mayor síntesis de la enzima (Quinto et al., 1977), y a una mayor concentración del RNAm específico de GS (Sánchez et al., 1978).

La GS de Neurospora de cultivos crecidos en glutámico como fuente de nitrógeno, purificada a homogeneidad, se estructura en un octámero con un peso molecular de 385,000; por otro lado, en cultivos limitados de nitrógeno, la forma oligomérica predominante es la de un tetrámero, (Limón-Lason et al., 1977), misma que se encuentra en las cepas auxótrofas parciales de glutamina en Neurospora (Dávila et al., 1978).

Recientemente se reportó la participación de dos polipéptidos diferentes en la molécula de GS en Neurospora (Sánchez et al., 1980). Estos polipéptidos denominados α y β, se estructuran como tetrámeros y octámeros respectivamente (Dávila et al 1980).

INTRODUCCION AL PROYECTO DE INVESTIGACION

El haber logrado un crecimiento controlado del hongo Neurospora crassa en cultivos limitados de nitrogeno o de carbón, permitió avanzar en el estudio de la regulación, la estructura y la composición de la glutamino sintetasa.

Por un lado se demostró que en condiciones de limitación de amonio, la glutamino sintetasa estructurada en un tetrámer con una alta actividad específica, no es regulada por el amonio per se, sino que la acumulación de esqueletos de carbón en estas condiciones y la baja actividad de glutamino sintetasa cuando la fuente de carbón es limitante, sugieren un efecto positivo de este compuesto sobre la regulación de esta enzima (Limón-Lason y col. 1977). Por otro lado la participación de dos polipéptidos diferentes en la estructura de la glutamino sintetasa nos permitió definir la composición del tetrámero de GS presente tanto en la limitación de amonio como en las mutantes auxotrofas de glutamina, y la composición del octamero de esta enzima que se presenta en el crecimiento exponencial del hongo cuando la fuente de nitrógeno no es limitante (Dávila y col. 1980).

El hecho de que la cepa am-1 de Neurospora que carece de la deshidrogenasa glutámica biosintética, creciera igual que la cepa silvestre en cultivos limitados de amonio, permitió la búsqueda y determinación de la glutamato sintasa, así como su regulación (Hummelt y col. 1980). Esto definió en Neurospora crassa la exsistencia de las dos vías de asimilación de amonio antes mencionadas.

La participación de dos monómeros diferentes en la actividad de la glutamino sintetasa, nos llevo a estudiar la regulación y la función de cada uno de ellos. El trabajo aquí presentado, demuestra que la vía que participa en la asimilación de amonio a altas concentraciones es a través de la deshidrogenasa glutámica y la forma octamérica de la glutamino sintetasa compuesta principalmente por el monómero β , mientras que en condiciones de limitación de amonio, la glutamino sintetasa estructurada en un tetrámero y compuesta principalmente por el monómero α es la primera enzima que fija amonio en la célula, y que la síntesis de acido glutámico se lleva a cabo por la actividad de la glutamato sintasa.

Finalmente, la síntesis <u>in vivo</u> de los monómeros α y β de la glutamino sintetasa en diferentes condiciones nitrogenadas correlaciona con los niveles de RNAm específico de cada monómero.

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PUBLICACIONES

REGULATION OF GLUTAMINE SYNTHETASE IN FED-BATCH CULTURES OF NEUROSPORA CRASSA.

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Summary

The effect of the nitrogen and carbon sources in the regulation of glutamine synthetase has been studied in fed-batch cultures of Neurospora crassa. The limitation of ammonium in an excess of the carbon source, leads to an accumulation of α-ketoglutarate and elevation of glutamine synthetase. The limitation of sucrose in an excess of ammonium results in a decrease in glutamine synthetase activity. These results indicate that the carbon source exerts a positive control in the regulation of glutamine synthetase.

Introduction

It is known that in bacteria α-ketoglutarate and glutamine regulate the activity of glutamine synthetase (EC 6.3.1.2) (1). Although the effect of different nitrogen sources on the activity of this enzyme has been studied in eukaryots (2, 3), the difficulties encountered in growing filamentous fungi under conditions of regulated growth, as in a chemostat (4, 5), have not made possible to evaluate the effect of limiting concentration of the carbon and nitrogen sources in the regulation of glutamine synthetase, as has been done in prokaryotic organisms (6). We have been able to regulate the growth of Neurospora crassa by continuously adding to the medium the limiting nitrogen or carbon source. This instrumentation, known as fed-batch culture (7), has allowed us to study the effect of the carbon and the nitrogen sources on the activity and oligomeric state of the enzyme glutamine synthetase.

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Material and Methods

Strains and Chemicals

Neurospora crassa wild-type strain 74-A and the glutamic acid dehydrogenase defficient mutant am-1, obtained from the Fungal Genetics Stock Center at the Humboldt State University Foundation, Arcata, Calif. U.S.A. All chemicals used were analytical grade.

Growth Conditions

Batch cultures of N. crassa were grown after inoculating conidia in Vogel's minimal medium (8) with 25 mM NH_4Cl as nitrogen source and 1.5% sucrose as carbon source. Fed-Batch cultures were achieved for up to 24 hs by pumping the limiting substrate (9.2 mM NH_4Cl or 14.6 mM sucrose) at a dilution rate of 3.2×10^{-3} hr^{-1}, into an agitated and aerated reactor vessel held at 25°C containing Vogel's medium lacking the nitrogen or carbon source used as limiting substrate. The NH_4Cl was pumped after incubating the conidia for 3 hs in Vogel's without nitrogen source, and the sucrose after germinating the conidia 4 hs in substrate excess. Under these conditions the change in reactor volume is negligible and it can be shown that the growth rate is constant and equals the product of the dilution rate, the feedstock concentration and the substrate yield constant.

Growth was determined by collecting mycelium samples on 0.45 μ membrane filters, washing with distilled water and placing in 5% trichloro acetic acid. After centrifugation the acid precipitate was resuspended in 1.0 N NaOH and protein was determined by the method of Lowry et al (9), using bovine serum albumin as standard.

Preparation of soluble extracts for amino acid analysis

Mycelium samples were harvested by filtration. Glutamic acid and glutamine were extracted by homogenizing the cells in 80% ethanol in the presence of L-U-^{14}C glutamine and L-U-^{14}C glutamic acid, separated using the method of Yemm and Cocking (10) as modified by Ferguson and Simms (11), and quantified by isotopic dilution. α-ketoglutarate was assayed accordingly to Bergmeyer and Bernt (12), after resuspending the washed mycelium in 0.6 N $HClO_4$ and neutralizing the supernatant with K_3PO_4.

Determination of glutamine synthetase activity

Glutamine synthetase measured as transferase activity was assayed as described by Ferguson and Simms (11). Units of activity are expressed as micromoles of γ-glutamyl hydroxamate produced per min at 30°C per mg of protein.

Sucrose Gradient Sedimentation

The samples, in a final volume of 0.3 ml were layered over a 5 to 20% continuous sucrose gradient and centrifuged at 4° for 12 hs at 248,000 x g in the Beckman SW 40 rotor. After centrifugation, fractions were ob-

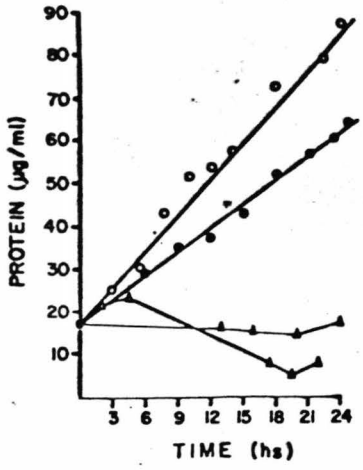

Fig. I Growth curves of the wild-tipe strain 74A in limiting ammonium (●) or sucrose (○) in fed-batch culture. Without ammonium (▲) or sucrose(△) also shown.

ıined from the top of the tube and glutamine synthetase activity was deter ıined in each fraction. Details are given in ref. 13.

esults

Neurospora crassa wild-type 74-A exhibits exponential growth with doubling time of 2 to 3 hs, when grown under conditions of excess substrate. However, in fed-batch culture linear growth is found as shown in g. 1.

Under conditions of ammonium limitation we found, after 12 hs, an lmost 12 fold increase in the intracellular concentration of α-ketoglutara e, while glutamine and glutamate decrease ten and two times, respective y, compared to the control growing in excess substrate. However, when ucrose is the limiting substrate, α-ketoglutarate does not vary appreci- bly and glutamine decreases three times (Table 1).

Under ammonium limitation a ten-fold rise in glutamine synthetase ctivity is found. However, when sucrose is limiting the activity of this

TABLE 1.- Intracellular concentrations of α-ketoglutarate, glutamate and glutamine, and glutamine synthetase activity in the wild-type strain 74-A, under nitrogen or carbon source limitation*.

Condition	hs	kg	glu	gln	G.S.
Batch, ammonium plus carbon excess	12	0.002	0.183	0.480	0.09
Fed-batch, ammonium limited	12	0.025	0.069	0.040	0.42
	18	0.020	0.084	0.045	0.77
	24	0.015	0.049	0.040	0.99
Fed-batch, sucrose limited	12	0.003	0.169	0.320	0.02
	18	0.003	0.120	0.270	0.02
	24	0.002	0.145	0.190	0.02

*α-ketoglutarate (kg), glutamate (glu) and glutamine (gln) concentrations expressed as μmoles/mg of protein. Glutamine synthetase activity (G.S.) expressed as μmoles of product per min per mg of protein.

enzyme falls four times compared to that found in an excess of substrate (Table 1). To ascertain whether these effects are due to ammonium per se or are mediated by the trapping of α-ketoglutarate and/or its conversion to glutamate or glutamine, the mutant strain am-1, which lacks the activity of the biosynthetic glutamic acid dehydrogenase and grows very slowly in excess ammonium, was grown under the same conditions. We found that this strain, when limited of ammonium, has the same growth rate as the wild-type grown under the same conditions. The activity of glutamine synthetase and the intracellular concentrations of α-ketoglutarate and glutamate are quite similar to those found in the wild-type strain; glutamine is not detectable in comparison with the wild-type, where this amino acid is just in the limits of detection. Furthermore, when there is an excess of ammonium present, the above parameters still resemble those found in the wild-type in ammonium limitation (Table 2).

When the wild-type strain is grown in glutamate as the nitrogen

TABLE 2.- Intracellular concentrations of α-ketoglutarate, glutamate and glutamine, and glutamine synthetase activity in the mutant strain am-1, under nitrogen limitation*.

Condition	hs	kg	glu	gln	G.S.
atch, ammonium plus carbon excess	12	0.012	0.027	0.045	0.55
Fed-batch, ammonium limited	12	0.023	0.031	N.D.	0.62
	18	0.022	0.055	N.D.	0.86
	24	0.020	0.079	N.D.	0.81

Abbreviations as in Table I. Not detected (N.D.)

source, glutamine synthetase is found as an octamer (13). We have found that a lower oligomeric form of this enzyme, possibly a tetramer, is found in the wild-type strain when ammonium is limiting, but not when it is in excess concentration (fig. 2). The mutant strain am-1, however, exhibits only the lower oligomeric state, regardless of the way ammonium is administered (data not shown).

Discussion

It is not possible to regulate the growth rate of Neurospora crassa in batch cultures just by changing the substrate concentration. However, this has been achieved by the use of fed-batch cultures.

We have shown that glutamine synthetase activity rises when ammonium is limited. This rise with time corresponds to the increase in limitation that occurs as result of growth, addition of substrate remaining constant. Coinciding with this effect, we found an important rise in α-ketoglutarate together with a decrease in glutamate and glutamine. We would like to propose that the carbon source exerts a positive control in the induction of glutamine synthetase in Neurospora crassa since: a) ammonium does not

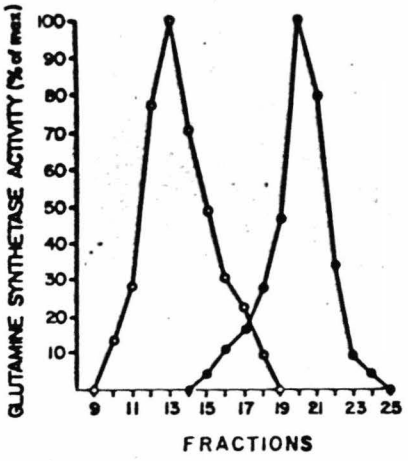

Fig. 2 Sedimentation in sucrose gradients of glutamine synthetase from the wild-tape strain 74A grown in limiting (○) and excess (●) ammonium.

have an effect per se, as shown by the increase in enzyme activity in the glutamic acid dehydrogenase deficient mutant grown in excess ammonium, as compared with the wild type and b) by the decrease of the activity under sucrose limitation in the wild-type strain where ammonium is in excess. This data indicates that ammonium does not exert its main effect through its conversion to glutamine, for if this were the case, a rise in activity would also be expected when carbon is limiting and glutamine falls (Table 1). It is suggestive that a rise in α-ketoglutarate is found whenever the enzyme is induced (Tables 1 and 2). This elevation is to be expected if ammonia is limited or if a block exists in the conversion of ammonium and α-ketoglutarate to glutamic acid. On the other hand we have recently reported that an excess of ammonium or glutamine represses the de novo synthesis of glutamine synthetase (4).

Very possibly a relationship exists between the oligomeric state of glutamine synthetase and its capacity to fix ammonium at low or high sub-

rate concentrations. The ability of the tetramer to synthesize glutamine low substrate concentration is being studied.

The fact that a strain completely lacking the biosynthetic glutamic id dehydrogenase grows as well as the wild-type in limited ammonium akes worthy to look for the activity of glutamate synthase in Neurospora assa. The catabolic glutamic acid dehydrogenase has low affinity for mmonium (15), and thus cannot be expected to be fixing ammonium under ese conditions.

The results clearly show the advantage of the use of growth controlled ltures in the study of enzyme regulation in filamentous organisms.

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RELATION BETWEEN STRUCTURE AND FUNCTION OF Neurospora crassa GLUTAMINE SYNTHETASE

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Summary

Two distinct monomers, α and β participate in the structures of different oligomers of Neurospora crassa glutamine synthetase (EC 6.3.1.2). In ammonium-limited cultures a tetrameric form composed mainly of α monomers was found. In excess of nitrogen an octameric form composed mainly from β monomers is the predominant oligomeric state. The presence of both monomers was observed in intermediate oligomeric forms.

Introduction

The nitrogen source regulates the concentration (1) and the de novo synthesis of glutamine synthetase (GS) in Neurospora crassa (2). On glutamate as nitrogen source the rate of synthesis of GS is 10-fold higher than in glutamine and corresponds to a similar difference in the specific mRNA levels of the enzyme (3). N. crassa GS purified from mycelia grown on glutamate is structured in an octameric form composed of monomers with a molecular weight of 48,000 (4). In Fed-batch ammonium-limited cultures the activity of GS increased and instead of an octamer, mainly a tetrameric form of GS was found (5). This lower oligomeric state of GS was also found in glutamine auxotrophs of Neurospora (6). Since these mutants grow in limited ammonium, it is possible that the two oligomeric states of GS correspond to different gene products (6), and have a different function in ammonia fixation.

Recently it has been found that two different monomers contribute to the activity of GS (Sánchez, et al; submitted for publication, 1979). These

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monomers can be separated in acrylamide gel electrophoresis in SDS-urea where one of them (α) runs slightly slower than the other (β).

In this paper we report the relation that exists between these monomers with the oligomeric state and the function of GS in Neurospora crassa.

Material and Methods

Strains and Chemicals. - Neurospora crassa wild-type strain 74-A, the glutamic acid dehydrogenase deficient mutant am-1 and the glutamine auxotroph gln-1a, were obtained from the Fungal Genetics Stock Center at the Humboldt State University Foundation, Arcata, Calif. U.S.A. The glutamine auxotroph gln-1c was obtained in our laboratory and is an allele of the auxotrophs previously reported (6). All chemicals used were analytical grade.

Growth Conditions. - Batch cultures of N. crassa were grown after inoculating conidia in Vogel's minimal medium (7) with 1.5% sucrose. The nitrogen source was glutamate at 25°C, or 37°C and glutamine at 37°C. Fed-batch ammonium-limited cultures at 25°C with a constant growth rate, were achieved as reported (5), except that the conidia were not previously incubated in the absence of nitrogen. Growth was determined as described (1).

Determination of Glutamine Synthetase Activity. - Glutamine synthetase measured as transferase activity was assayed as described by Ferguson and Sims (8) in cell-free extracts of Neurospora prepared as in a previous work (1).

Immunoprecipitation of in vivo-labeled Glutamine Synthetase. - The cultures were pulsed with [^{3}H] leucine for one hour before harvesting. The cell-free extracts were sedimented in sucrose gradients (4) and fractions of the principal peaks of activity were precipitated with $(NH_4)_2SO_4$ at 70% saturation, resuspended and dialyzed overnight. Aliquots were immunoprecipitated with antibody against GS as described (2).

Electrophoresis and Fluorography. - The immunoprecipitates were subjected to acrylamide slab gel electrophoresis in the presence of SDS and 7 M urea (Sánchez, et al; submitted for publication, 1979), stained with Coomassie blue (9) and treated for fluorography (10).

Purification of Glutamine Synthetase. - Conidia obtained from slants with glutamine (10 mM) as nitrogen source, were used to inoculate cultures with glutamate at 25°C. After 3 hs the germinated conidia were filtered, washed and dried with acetone (1). From this powder the octameric GS was purified as previously reported (4). The tetrameric GS from the glutamine auxotroph gln-1c was purified as reported (4) from cultures grown on glutamate at 37°C, except for the following modifications. The cell-free extract was prepared in buffer A diluted 10-fold, this buffer was used to equilibrate the DEAE-cellulose column, which was eluted with Buffer A (Buffer A contains 50 mM imidazole, 50 mM glutamic acid, 80

mM $MgSO_4$, 50 mM K_2SO_4, 0.5 mM EDTA, 5 mM 2-mercaptoethanol, 25 mM $NaHSO_3$, pH 7.2). The fractions with activity were pooled and made 2.25 mM $MnCl_2$ and after added to the sepharose-anthranilic acid column previously equilibrated with Buffer A 2.25 mM $MnCl_2$, the enzyme was eluted with 40 mM AMP in this buffer. The protein was precipitated with $(NH_4)_2SO_4$ at 70% saturation and resuspended and dialyzed against Buffer A.

Results and Discussion

To study the distribution of the two different monomers of GS in the different oligomeric forms of this enzyme, the wild-type 74-A strain was grown on batch cultures at 25°C with glutamate as nitrogen source and in Fed-batch cultures ammonium-limited. In these conditions the GS is mainly found as an octamer (1) and tetramer (5), respectively, In addition the 74-A strain and the gln-1c mutant strain were grown in glutamine

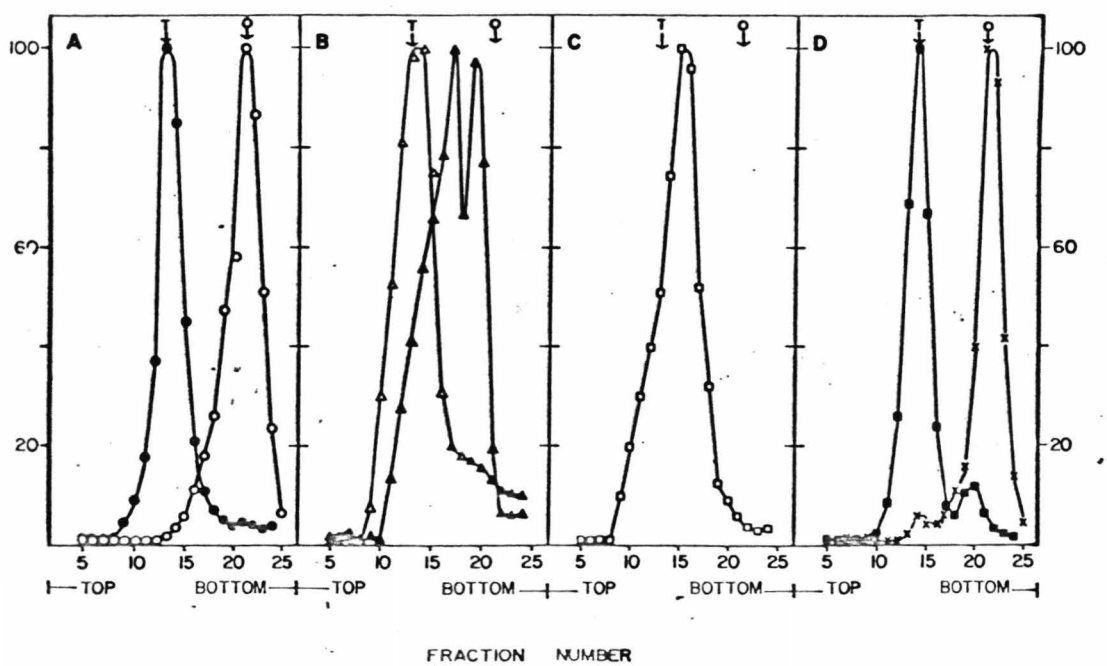

Fig. 1. Sucrose gradient sedimentation of glutamine synthetase from crude extracts: A, wild-type grown at 25°C on 5 mM glutamate (o) or ammonium-limited (●) B, at 37°C on 5 mM glutamine the wild-type (▲) and the mutant strain gln-1c (△) and C, the am-1 strain at 25°C ammonium-limited (□). D, purified GS from cultures grown on glutamate at 25°C from the wild-type (x) and from the gln-1c at 37°C (■). T tetramer, O octamer.

at 37°C and the am-1 mutant strain in limited-ammonium. The glutamine auxotrophs were grown on glutamine at 37°C since in this condition they had a sizable activity of GS structured in a tetramer (6). All the cultures were pulsed with [^{3}H]leucine as described in methods.

In Fig. 1 is presented the transferase activity in sucrose gradients from extracts of these cultures. As expected the tetrameric GS was present in extracts of the wild-type ammonium-limited and in the gln-1c (Fig. 1 A,B), the octamer was found in the 74-A strain grown on glutamate (Fig. 1 A). On the other hand in the wild-type grown on glutamine at 37°C, in addition to the octamer, a well defined peak of activity was found in the sucrose gradient left and next to this oligomer (Fig. 1 B). A different oligomer of GS, that banded next and right to the tetramer, was also found in the am-1 mutant strain ammonium-limited (Fig. 1C). The bands of activity immunoprecipitated, stained with Coomassie blue after acrylamide gel electrophoresis in SDS-urea, are shown in the top of Fig. 2. It is clear that the octamer is composed mainly by β monomers (Fig. 2c) and the tetramer by α monomers (Fig. 2d). Both monomers were found in the intermediate oligomeric forms that banded in sucrose gradient between the tetramer and the octamer (Fig. 2a,b). As these oligomers (am-1 strain and wild-type grown on glutamine at 37°C) band closer to the octameric form, an increase in the β monomers and a decrease in the α monomers was apparent. Only in the case of the wild-type strain grown in glutamine at 37°C the monomers corresponded to the pool of two peaks of activity of the sucrose gradient.

The fluorography of the gel (Fig. 2 bottom) shows that the distribution of α and β monomers resembles very closely what is seen after staining the gel (Fig. 2 top), and emphasizes that the newly synthesized

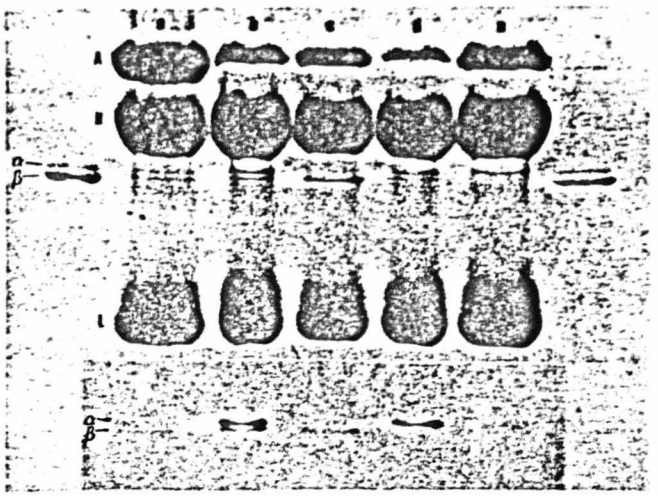

Fig. 2. Top panel Gel after coomassie-blue staining: a) wild-type on glutamine, b) am-1 strain ammonium-limited, c) wild-type on glutamate, d) wild-type ammonium-limited and e) gln-1c strain on glutamine. In the first and the last tracks of the gel purified non-labeled GS from glutamate grown cultures was run as a marker. For other conditions see Fig. 1 and methods. Bottom panel: gel after fluorography. A albumin, H γ-globulin heavy chain, L γ-globulin light chain, α and β monomers of GS.

α's are incorporated almost exclusively in tetramers and the β's in octamers. In contrast, hybrid monomers were found in the oligomers intermediate between the tetramer and the octamer.

It has been possible to obtain growth conditions in which the octamer can be purified with only traces of α monomers (compare Fig. 2 and 3). These data indicate that the α monomers are not required for the structure and activity of the octamer. The tetramer purified from gln-1c mutant strain is composed only of α monomers (Fig. 3).

The presence of some α monomers in the octamer and of some β monomers in the tetramer, of the wild-type strain may be the result of monomer hybridization, or that the equilibrium of α's favors more the arrangement in tetramers than in octamers, and viceversa for the β's. The fact that the α monomers of the mutants strain gln-1a and gln-1c in

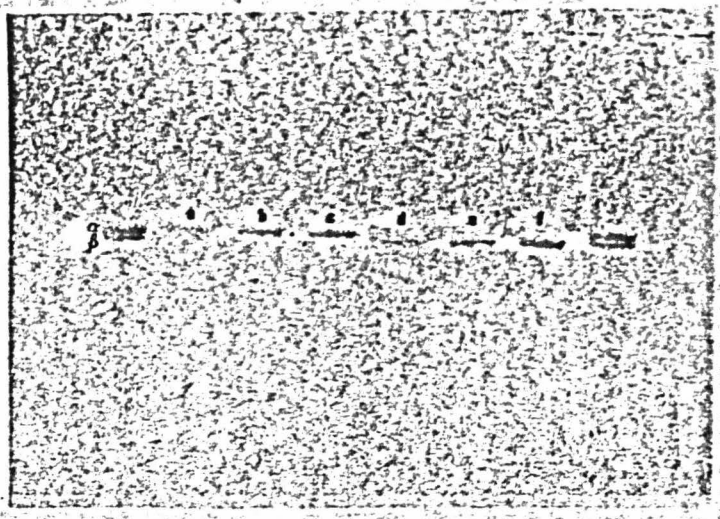

Fig. 3. Gel after coomassie-blue staining of the purified preparations of GS obtained from gln-1c strain (a, b and c) and from wild-type (d, e and f). The cultures were grown as described in methods. In the first and the last traks a mixture of α and β monomers were run as markers.

addition to the tetramer, are able to form octamers in a low proportion, and that the monomers of the wild-type strain behave oppositively (Fig. 1d), is in favour of the equilibrium hypothesis. This equilibrium would also depend of the intracellular conditions. It is in intermediate oligomeric forms of GS where both monomers are found in an important proportion, then it is possible that these intermediate states are composed by hybrids of α and β monomers, which appear when the nitrogen content is neither limiting nor in excess. Experiments are in progress to purify the wild-type tetramer and test in vitro if the presence of this form in low ammonium and the octamer in nitrogen excess, are related with a different affinity for ammonium of these oligomers. Recent evidence has demonstrated that glutamate synthase (GOGAT) is present in ammonium-limited cultures (Hummelt, et al; submitted for publication, 1979). This enzyme together with the tetrameric GS participates in the assimilation of low ammonium concentrations.

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Title:

PHYSIOLOGY OF NITROGEN ASSIMILATION IN *NEUROSPORA CRASSA*.

Running title:

NITROGEN ASSIMILATION IN *NEUROSPORA*.

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INTRODUCTION:

Folkes and Sims (10), using ^{15}N-labeled ammonium, demonstrated that in Candida utilis the glutamate dehydrogenase (GDH) (E.C.1.4.1.4.), and the glutamine synthetase (G.S.) (E.C.6.3.1.2.) activities are responsible for ammonium assimilation. Studies in other fungi such as Schizosaccharomyces have shown that in some species of this genus, GS is the first enzyme to fix ammonium in low concentrations; the glutamine formed is converted into 2 molecules of glutamate by the action of a glutamate synthase (GOGAT) (E.C.1.4.7.1.) in the presence of 2-oxoglutarate and NADH (4). GOGAT activity has also been found in Saccharomyces cerevisiae but in very low amounts and its significance is unclear (20). In plants the fixation of low amounts of ammonium by the GS-GOGAT patway is very well established (14).

Neurospora crassa growing on high ammonium concentration has an elevated biosynthetic GDH activity; a mutant has been described that lacks this activity and grows poorly in ammonium excess (9). We found that this mutant grows as well as the wild-type Fed-batch ammonium limited cultures (11). An elevated NADH dependent GOGAT activity was found in either the wild-type or the mutant under ammonium limitation. Glutamate or Glutamine repress this enzyme (12). This data indicate the existence of the GS-GOGAT pathway in Neurospora, that functions under nitrogen limitation conditions.

In Neurospora the nitrogen source regulates the concentration (23), and the de novo synthesis of GS (19). In glutamate as nitrogen source the rate of synthesis of GS is 10 fold-higher

than in glutamine and corresponds to a similar difference in the level of specific mRNA that codes for the enzyme (21).

An octameric GS has been purified from cultures of the wild-type strain 74-A of Neurospora grown on glutamate as nitrogen source (17). On the other hand a tetrameric GS has been reported to be present in the wild-type strain grown on fed-batch ammonium limited cultures (13), as well as in partial glutamine auxotrophs of Neurospora (6). Recently we reported that in Neurospora crassa GS is composed of two different polypeptides called α and β, structured in a tetramer and an octamer, respectively (6), (22).

In this paper we report in Neurospora, the growth conditions that regulate the in vivo synthesis of one or another GS monomer, the levels at which this regulation is exerted, and the participation of the α and β GS polypeptides in different ammonium assimilation pathways.

MATERIAL AND METHODS:

Strains. Neurospora crassa wild-type strain 74-A, was obtained from the Fungal Genetics Stock Center at the Humboldt State University Foundation, Arcata, Calif. U.S.A. The glutamine auxotroph gln-1c was obtained in our laboratory and is an allele of the auxotrphs previously reported (6).

Growth conditions. Batch cultures of Neurospora were grown after inoculating conidia in Vogel's minimal medium with 1.5% sucrose. The nitrogen sources were 25mM NH_4Cl, 5mM glutamate or 5mM glutamine. Ammonium limitation in fed-batch cultures was achieved as has been described (13).Growth was determined as described (23).

Enzyme activities. Glutamine synthetase activity measured as transferase was assayed as described by Ferguson and Sims (8), in cell-free extracts of Neurospora prepared as in a previous work (23). Glutamate dehydrogenase activity was measured following NADPH oxidation as described by Fincham (9). Glutamate synthase activity was measured by the method of Boland and Benny (2). Cell-free extracts to measure these enzymes were prepared from acetone dried powders, disrupted in a Braun-Cell homogenizer MSK type 853030 in the presence of 0.1M potassium phosphate buffer (pH 7.6).

Sucrose gradient sedimentation. The samples in a final volume of 0.4ml were layered over a 5 to 20% continuos sucrose gradient and centrifuged at 4°C for 12 h at 248,000xg in the Beckman SW-40 rotor; after centrifugation fractions were obtained from the top of the tube and glutamine synthetase activity was determined in each fraction.

In vitro mRNA translation. Polysomal RNA was prepared as previously described (21). RNA fractions were translated in a reticulocyte lysate system in the presence of ^{35}S methionine, after incubation the reaction was stopped and glutamine synthetase was immunoprecipitatted by a direct procedure using 1μg of purified wild-type glutamine synthetase as carrier and 1.5mg of specific antibody. Immunoprecipitates were separated by centrifugation through sucrose gradients as previously described (18). The immunoprecipitates were subjected to acrylamide slab gel electrophoresis in the presence of SDS and 7M urea (22), and treated for fluorography (3).

Immunoprecipitation of in vivo labeled glutamine synthetase. The cultures were pulsed with ^{3}H leucine for 1 h before harvesting, the cell-free extracts were immunoprecipitated with antibody against glutamine synthetase as described (19), and the samples were subjected to electrophoresis and fluorography as indicated above.

Electrophoresis in agarose gels containing methylmercuric hydroxide.

Gel electrophoresis of mRNA was performed in vertical slab gels cast with 1% agarose, supplemented with 5mM CH_3HgOH as described by Bailey and Davidson (1). Samples were supplemented with 10mM CH_3HgOH. After electrophoresis RNA was visualized by ultraviolet light illumination (1). Size fractionation of RNA was carried out in these gels, and RNA was eluted as described by Dunn et al (7).

RESULTS

GS purification from the wild-type strain grown under ammonium limitation.

We have previously reported the regulation by nitrogen of the synthesis and mRNA concentration specific for GS (23, 19, 21), at that time it was assumed that this enzyme was composed by a single type of plypeptide. We have already communicated that cultures grown on glutamate, were the fungi grows exponentially with an elevated activity of GS, the octameric form of this enzyme is composed mainly by the β polypeptide. On the other hand under ammonium limitation where the fungi grows at a lower rate, the tetrameric form of GS is composed mainley by the α polypeptide. It has been also found that the β polypeptide purified from the wild-type strain grown on glutamate is arranged as an octamer, while the α polypeptide purified from a glutamine auxotroph is structured as a tetramer (6). Although in a low yield, we have been able to purify the GS from the wild-type growing on low ammonium and found as the major component, the tetramer and the α monomers, some amount of the octamer and the β monomers were also present (Fig. 1).

Oligomeric forms synthesis and in vitro translation of the specific mRNA of glutamine synthetase in nitrogen excess and limitation.

After we found that wo different oligomers, composed of distinct polypeptides were responsible for GS activity (22, 6), the relation between the synthesis and the mRNA concentration of the two GS polypeptides with the oligomeric form, was reinvestigated in different nitrogen conditions during the growth phase. The β polypeptide of GS was preferentially synthesized

when Neurospora was growing exponentially on an excess of ammonium, glutamate or glutamine, although some α monomers were also synthesized. The β polypeptide was synthesized at a higher rate on glutamate or ammonium than on glutamine, which is in accordance with the enzime activity (Fig. 2 and Table I). We have previously reported that under these conditions GS is present as an octamer (23). In longer incubation times, when the cultures were approaching the stationary phase, both polypeptides were synthesized and the GS activity was found in a broader peak that resembles more a tetrameric than an octameric form (Fig. 2). When Neurospora grows under ammonium limitation, the polypeptide is preferentially synthesized (Fig. 2).

The in vitro translation of mRNA specific for the α and β monomers showed a correlation with its synthesis in all the conditions studied (Fig. 2).

Different mRNA codify for two polypeptides of GS ?

The obtention of glutamine auxotrophs that lack the GS β polypeptide (6), and the fact that a precursor-product relation ship could not be demonstrated between α and β polypeptides in a cell-free translation system (22), suggests the participation of two mRNA in the synthesis of GS polypeptides.

In order to obtain a more direct evidence of the presence of one mRNA for each GS polypeptide, agarose gel electrophoresis of RNA in the presence of methylmercuric hydroxide was performed. Populations of mRNA from wild-type cultures grown on glutamate as nitrogen source for 12 h at 25°C were fractioned, and in vitro translated. Results obtained from three different experiments

showed that the β/α ratio of in vitro synthesized polypeptides changes from different gel fractions. In fact, the main product from the heavier mRNA zone was the α monomer, while the β monomer was encoded in a ligther zone (Fig.3).

Ammonium assimilation enzymes during nitrogen shifts.

To better understand the role of the GS polypeptides on the assimilation of ammonium, Neurospora was grown on nitrogen excess for 6 h and shifted afterwards to ammonium limitation for 12 h. The GS oligomeric forms, the specific activity and the in vivo synthesis of the GS polypeptides before and after the shift are shown in Fig. 4 and in Table I. When the cultures were grown on nitrogen excess the octameric GS present was composed almost exclusively by the β monomers. After the shift the octamer was substituted by the synthesis of a tetrameric enzyme constituted mainly by the monomer, characteristic of ammonium limited cultures.

The activities of ammonium assimilation enzymes were measured before and after the shift. On ammonium excess a low GOGAT activity was present, the GDH was elevated and a moderate amount of GS activity was present. On glutamate and glutamine the activity of GOGAT was almost not detected, the GDH activity was lower than on ammonium and the GS activity was very high on glutamate, intermediate on ammonium and low on glutamine. After the shift to low ammoniu, the culture that was previously on high ammonium, grew linearly, however the cultures that were before on glutamate or glutamine presented a lag before growing linearly. When the cultures were shifted to ammonium limitation,

low GDH and elevated GS activity were found. The GOGAT activity increased 23, 1.5 and 8-fold in the cultures that came from ammonium excess, glutamate and glutamine, respectively Table I).

Similar studies were performed in cultures grown on limiting ammonium and shifted to an excess of nitrogen. Enzyme activities, synthesis of α and β monomers and GS oligomeric forms were analysed before and 12 h afrer the shift. After a 4 h lag the cultures began to grow exponentially on glutamate, ammonium or glutamine. At this time intracellular aminoacids pools were meassured in the culture shifted to ammonium excess glutamate had increased 2-fold and glutamine 40-fold. Twelve hours after the shift the ratio β/α polypeptide synthesis in the three nitrogen sources was higher than that present during nitrogen limitation. On the other hand the fact that on ammonium excess or glutamate the α monomer is synthesized at the same or higher rate than the β monomer, and the presence of a broader GS oligomeric form that migrates as a tetramer in the sucrose gradient (Fig. 5), is in accordance with the data of Fig. 2, where the α polypeptide was preferentially synthesized at longer incubation times. Figure 7 presents as experiment in which the synthesis of the GS monomers was measured at shorter incubation times after the addition of an excess of ammonium. It is clear that 6 h after the addition, only the β monomer was synthesized.

After the addition of ammonium the GDH activity increased 3-fold, and the GOGAT activity decreased 20-fold.

In distinction, the addition of glutamate and glutamine resulted in a moderate diminution of GDH activity and in a dramatic decrease in GOAT activity (Table 2). The activity of GS correlated with its synthesis; it was high on glutamate a little lower on ammonium and very low on glutamine (Table 2).

FIGURE LEGENDS

Figure 1. Purification of GS from the wild-type 74-A grown on limited ammonium. A; sucrose gradient sedimentation and B; Acrylamide SDS-urea gels stained with Coomasie-blue of the GS purified from the wild-type grown at 25°C under ammonium limitation. In A, the position of the tetramer (T) and the octamer (O) of GS is indicated. In B, gels of the purified fraction (2) as well as markers of the α (1) and the β polypeptide (3) are shown.

Figure 2. Growth, oligomeric forms and *in vivo* and *in vitro* synthesis of the GS from the wild-type 74-A strain. A; Fluorography of the GS region of acrylamide gels of immunoprecipitates obtained from *in vivo* labeled GS or *in vitro* mRNA translation; cultures were grown on: (1) limited ammonium 12 h; (2) 25mM ammonium 12 h; (3) 25mM ammonium 24 h; (4) 5mM glutamate 12 h; (5) 5mM glutamate 24 h; (6) 5mM glutamine 12 h and (7) 5mM glutamine 24 h. B; Growth and C; Sucrose gradient sedimentation from cultures grown on 25mM ammonium (●), 5mM glutamate (o) and 5mM glutamine (▲) for 24 h.

Figure 3. Fractionation of GS mRNA in methylmercuric hydroxide agarose gel electrophoresis. Polysomal RNA from the wild-type strain grown on glutamate at 25°C for 12 h was prepared and fractionated on oligo-dT cellulose to obtained the poly-A enriched fraction. This fraction was subjected to electrophoresis in the presence of methylmercuric hydroxide. RNA from fractions of the gel was eluted and *in vitro* translated, and GS

polypeptides were identified by immunoprecipitation followed by acrylamide gel electrophoresis and fluorography. Fluorographies and scanning are presented: a-c, three consecutive fractions of the gel from the region where GS mRNA moves; d, mRNA preparation before electrophoresis.

Figure 4. Changes in the in vivo synthesis and in the oligomeric form of GS from the wild-type strain cultures transfered from nitrogen excess (●) to ammonium limitation (o). A; Fluorography of immunoprecipitated obtained from in vivo labeled GS, and B; Sucrose gradient sedimentation of GS. Cultures were transfered from (a) 25mM ammonium 6 h, (b) 5mM glutamate 6 h, (c) 5mM glutamine 6 h to ammonium limitation for 12 h.

Figure 5. Changes in the in vivo synthesis and in the oligomeric form of GS from the wild-type strain cultures shifted from ammonium limitation to nitrogen excess. A; Sucrose gradient sedimentation of GS, B; Fluorography of immunoprecipitated obtained from in vivo labeled GS, and C; Growth curves from the wild-type strain grown on ammonium limitation for 12 h (●), and then shifted for 12 h to 25mM ammonium (o), 5mM glutamate (▲) or 5mM glutamine (Δ).

Figure 6. Fluorography of immunoprecipitated of in vivo labeled GS monomers from the wild-type cultures grown 12 h on limited ammonium (a) and then shifted to 25mM ammonium for 6 (b) and 12 h (c).

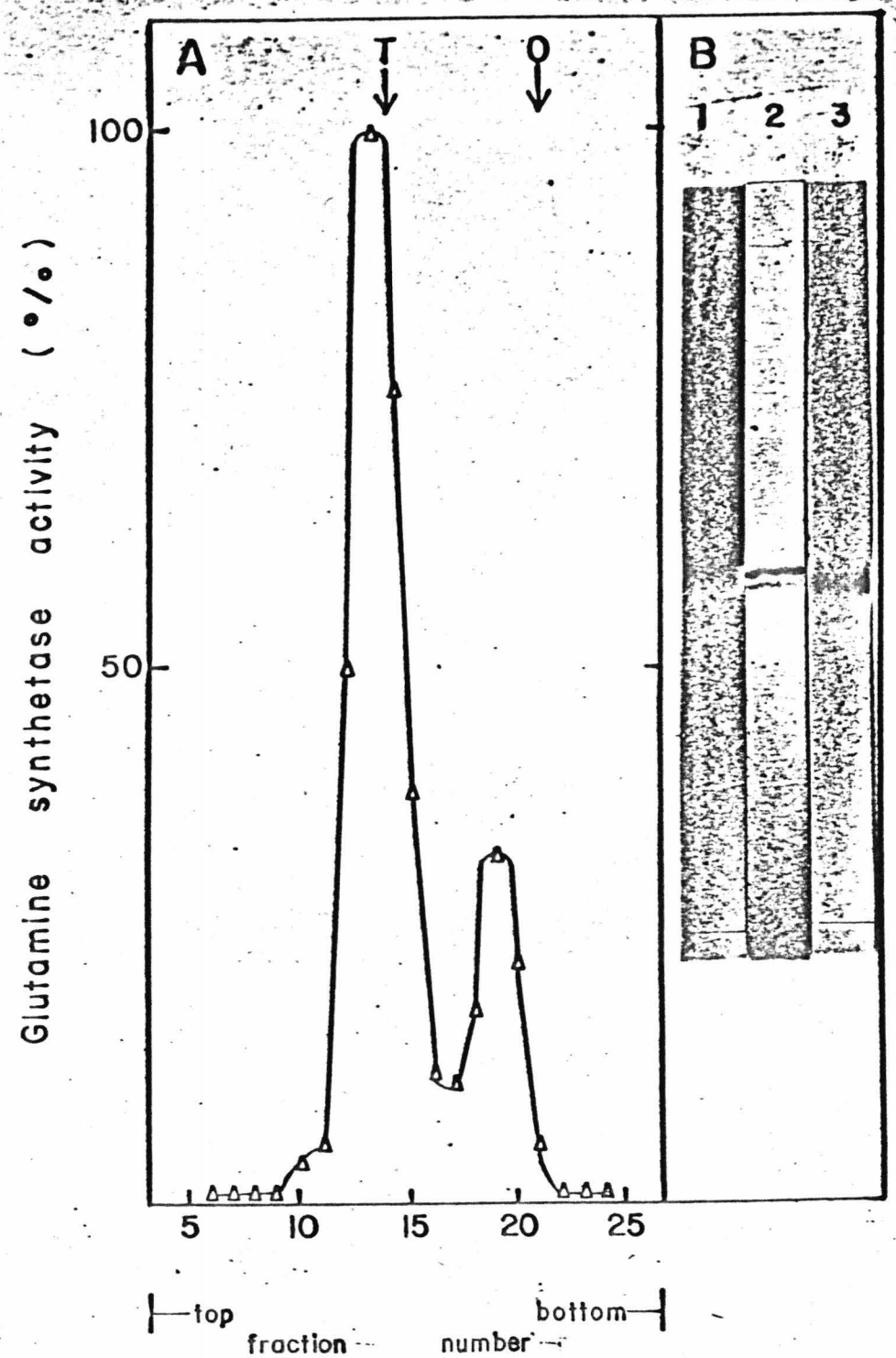
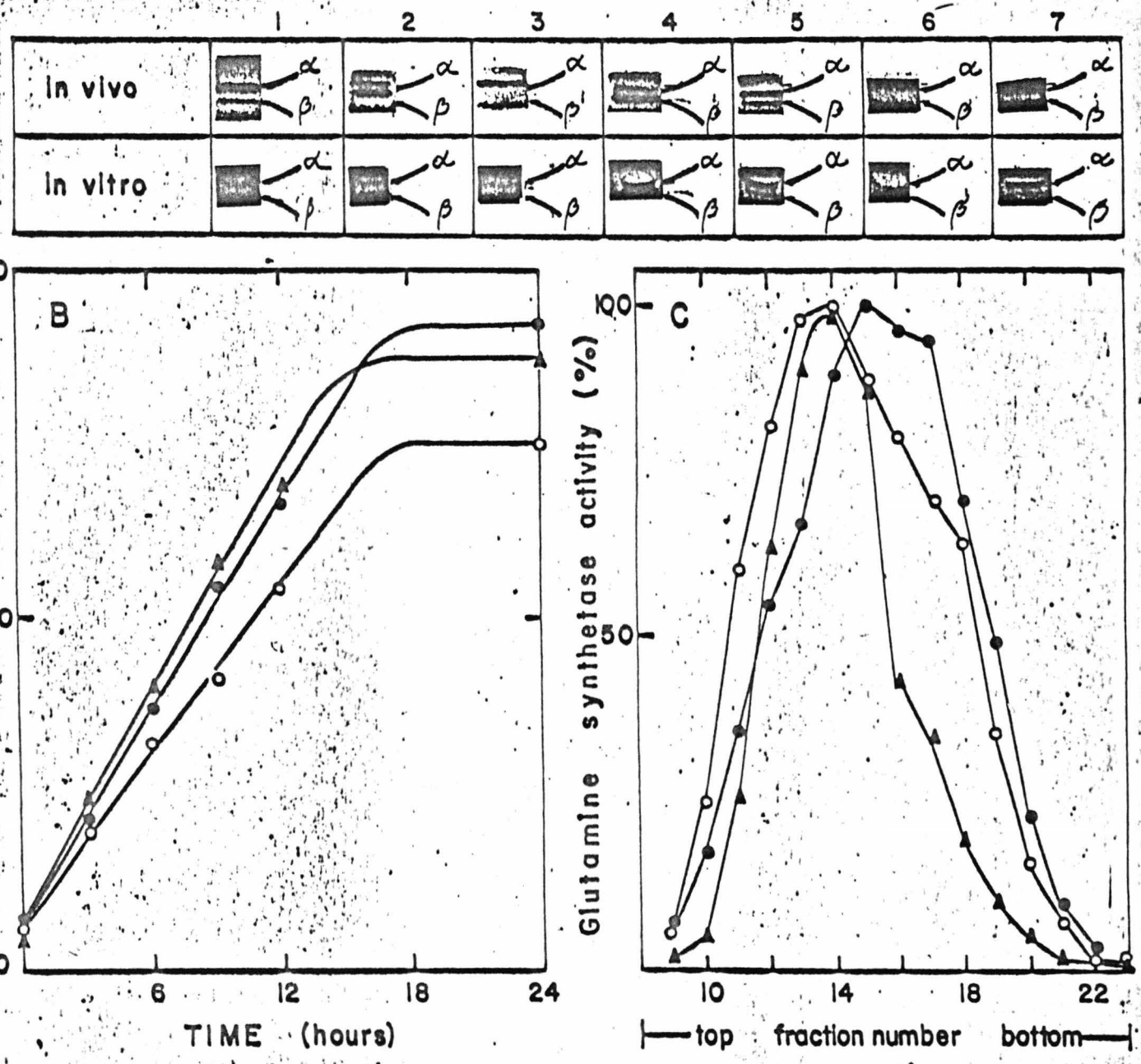

A
T
O
B
1
2
3
100
50
Glutamine synthetase activity (%)
5
10
15
20
25
top
bottom
fraction number

Figure 1.


A
1
2
3
4
5
6
7
In vivo
In vitro
α
β
β'
B
1000
100
10
Protein µg / ml
6
12
18
24
TIME (hours)
C
100
50
Glutamine synthetase activity (%)
10
14
18
22
top
fraction number
bottom

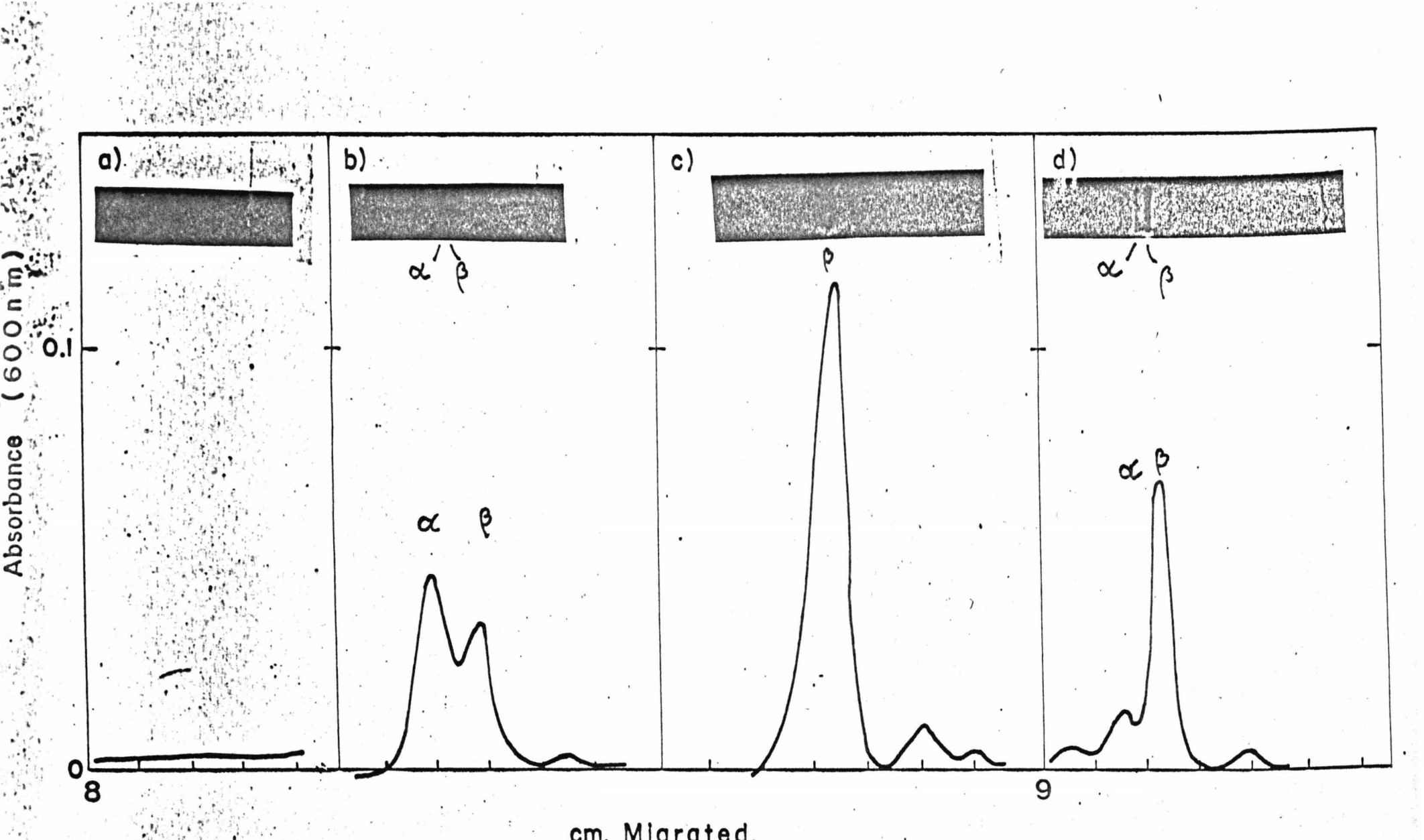


Figure 3.

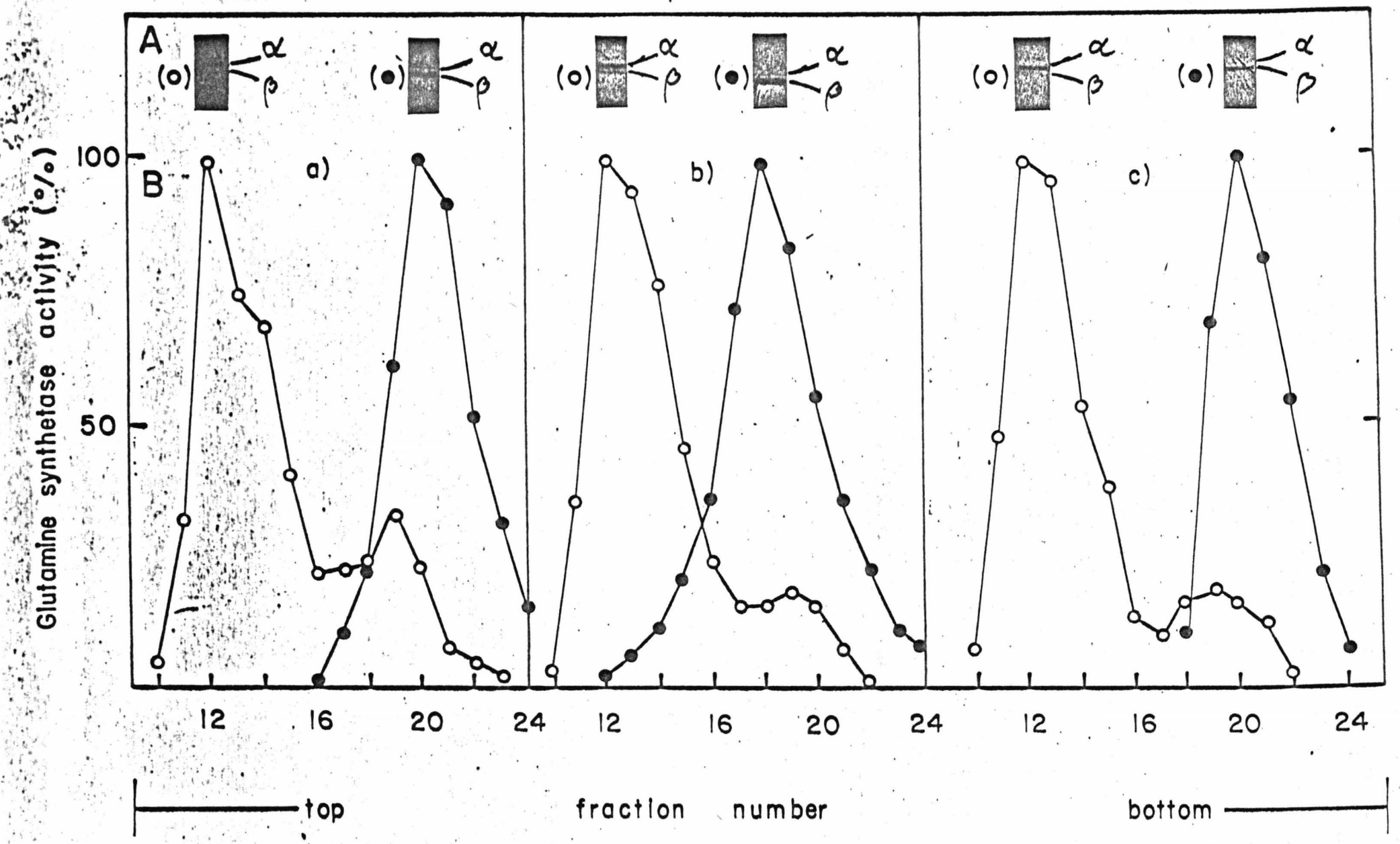

A
(o)
α
β
(•)
α
β
(o)
α
β
(•)
α
β
(o)
α
β
(•)
α
β
B
a)
b)
c)
100
50
Glutamine synthetase activity (%)
12
16
20
24
12
16
20
24
12
16
20
24
top
fraction number
bottom

Figure 4.

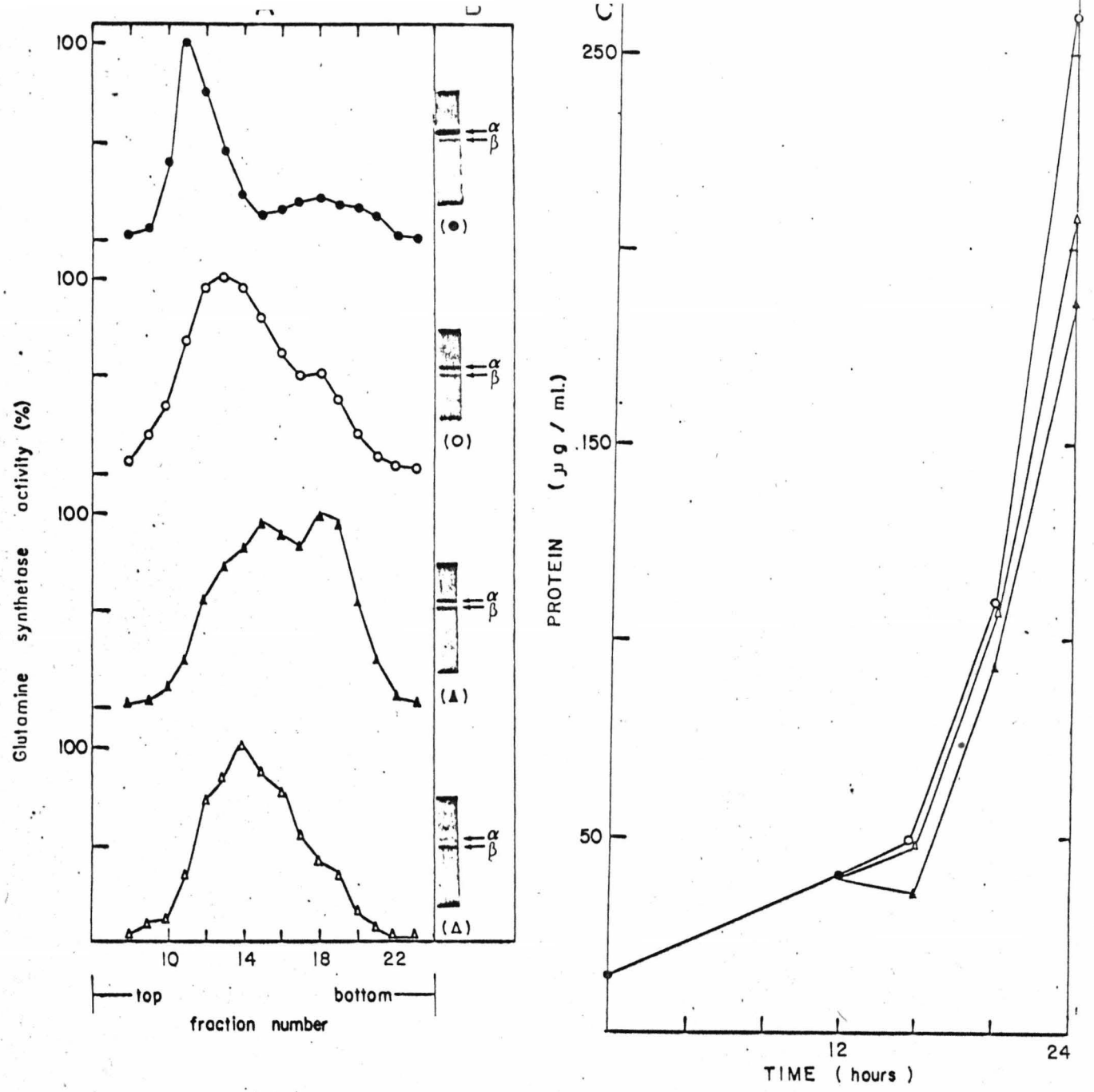

Glutamine synthetase activity (%)
100
100
100
100
(●)
(○)
(▲)
(△)
α
β
10
14
18
22
top
bottom
fraction number
PROTEIN (μg / ml.)
250
150
50
12
24
TIME (hours)

Figura - 5

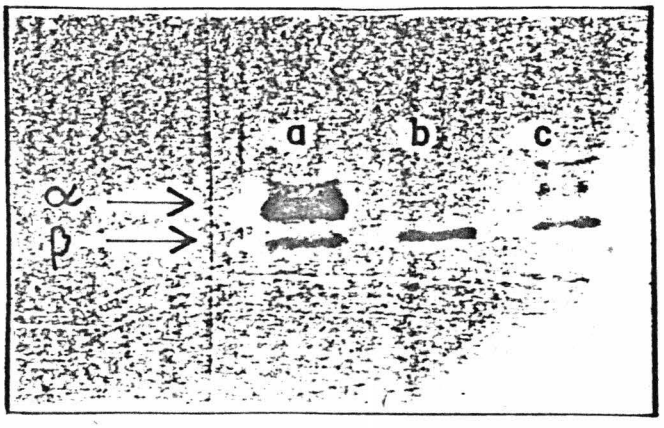


Figure 6.

Table 1. GS, GDH and GOGAT specific activities during nitrogen shifts.

Nitrogen shifts	Time (h)	GS U/mg	GDH U/mg	GOGAT U/mg
From ammonium excess to NH_4^+ limitation	6	0.07	0.1	0.002
	12	0.29	0.07	0.047
From glutamate to NH_4^+ limitation	6	0.25	0.05	0.001
	12	0.11	0.09	0.002
From glutamine to NH_4^+ limitation	6	0.02	0.05	0.002
	12	0.14	0.09	0.016

Table 2. GS, GDH and GOGAT specific activities during nitrogen shifts.

Nitrogen shifts	Time (h)	GS U/mg	GDH U/mg	GOGAT U/mg
From limited ammonium	12	0.24	0.06	0.027
to NH_4^+ excess	12	0.11	0.166	0.001
or glutamate	12	0.22	0.08	0.01
or glutamine	12	0.05	0.09	ND

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DISCUSION

Hemos reportado que la GS se estructura en dos formas oligoméricas diferentes, compuestas cada una por diferentes monómeros. El hecho de que no exsiste una relación precursor producto entre los monómeros α y β de GS en un sistema de traducción in vitro, y la separación de dos mensajeros específicos de GS, indica la participación de dos RNAm en la síntesis de GS y sugiere la exsistencia de dos genes de GS en Neurospora. Esta proposición se apoya en la exsistência de cepas auxotrofas parciales de glutamina que han perdido el monómero β de GS o lo tienen alterado. En algunas de estas cepas mutantes la actividad de GS se encuentra en un tetrámero compuesto por el monómero α. Ademas como se muestra en este tabajo, la síntesis del polipeptido α se regula de una manera diferente a la del polipeptido β, y sus respectivos oligómeros el tetrámero y el octamero, participan en diferentes vías de asimilación de amonio. La obtención de mutantes deficientes en el polipeptido α de GS nos dara evidencias definitivas de la exsistência de dos genes para los dos monómeros de GS en Neurospora.

La forma octamérica de GS compuesta por el polipeptido β esta presente durante el crecimiento exponencial de hongo, en un exceso de substratos. La síntesis del polipeptido β de GS es regulada por la fuente de nitrogeno, siendo la síntesis tanto in vivo como in vitro mayor en glutámico intermedia en amonio y muy baja en glutamina. La alta actividad de GDH y baja de GOGAT en un medio con exceso de amonio, sugiere que la síntesis de glutámico es a través de la GDH. Cuando glutámico es la fuente de nitrógeno en el medio, la actividad de GDH es muy ba-

ja y la actividad de GOGAT casi no se detecta. En glutamina como fuente de nitrógeno la actividad de las enzimas que participan en la asimilación de amonio es muy baja, lo que señala a este amino acido como el producto final de la vía.

En tiempos largos de crecimiento, el polipeptido α se empiesa a sintetisar estructurandose la GS en un tetrámero. La expresion del polipeptido α en estas condiciones coincide con la disminución en la velocidad de crecimiento característica de la fase preestacionaria, donde el substrato limitante es el oxigeno.

La determinación de las actividades de GS, GDH y GOGAT en cultivos que fueron cambiados a una limitación de amonio o a un exceso de nitrógeno nos permitió entender las vías de asimilación de amonio en Neurospora.

El hecho de que en exceso de amonio se sintetise preferentemente el polipeptido β de GS y que la actividad de GDH sea elevada en estas condiciones, indica que estas dos enzimas son las responsables de la asimilación de amonio en altas concentraciones. Esto correlaciona con el hecho de que la cepa mutante que carece de la actividad de GDH crece deficientemente en exceso de amonio.

Cuando los cultivos se transfieren a un medio limitado de amonio, disminuye la síntesis del polipeptido β mientras que la síntesis del polipeptido α y la actividad de GOGAT aumentan varias veces. La actividad de GDH disminuye ligeramente en estas condiciones. La baja afinidad por amonio de la GDH y el hecho de que la cepa que carece de esta enzima cresca igual que la cepa silvestre en condiciones de limitación de

amonio, hace poco probable la participación de la GDH en la vía de asimilación de bajas concentraciones de amonio. Ademas la cepa mutante que carece de la actividad de GOGAT, presenta una fase lag al crecer en cultivos limitados de amonio. Estos datos, establecen que la vía que asimila amonio a bajas concentraciones es a través de la forma tetramerica de GS compuesta por el monómero α y la actividad de GOGAT.

Con excepción de los cultivos transferidos de alto a bajo amonio, todos los demas presentan una fase lag a modificarse la condición nitrógenada. Se estan realizando experimentos a cortos tiempos que ayudaran a entender esta fase lag que requiere Neurospora para crecer cuando se modifican las condiciones nitrogenadas.

Independientemente de la fuente de nitrógeno presente en exceso, todos los cultivos cambian el octamero(β) por el tetrámero(α) de GS cuando son trnsferidos a cultivos limitados de nitrógeno. En los geles de SDS teñidos con Coomasie-blue de el inmunoprecipitado de GS, el polipeptido β no esta presente, lo que indica que este monómero presente en exceso de nitrógeno es degradado despues de que los cultivos se transfieren a bajas concentraciones de amonio. Se ha reportado la degradación de la GS en la cepa silvestre en condiciones de deprivación de carbón.

Finalmente se ha reportado en Neurospora el recambio de nitrógeno en condiciones de no crecimiento y la participación de GS en este proceso lo que indica que el amonio es reasimilado. Las vías que participan en la reasimilación y el significado funcional de este fenomeno requiere estudios posteriores.