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DIVISION DE ESTUDIOS DE POSGRADO

"ESTUDIOS SOBRE EL METABOLISMO NITROGENADO DE BOUVARDIA TERNIFOLIA"

TESIS

Que para obtener el grado de :

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P R E S E N T A

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Me es muy grato informar a usted que el alumno EZEQUIEL MURILLO GARCIA presentará próximamente su examen para obtener el grado de Doctorado en el área de Bioquímica ante el siguiente jurado.

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INTRODUCCION:

El nitrógeno, junto con el carbono está en el centro del metabolismo intermediario de una planta. A diferencia del carbono, el nitrógeno puede encontrarse en el ambiente en diferentes estadios de óxido reducción (NO_3, N_2, NH_4) . El sistema metabólico de la planta puede tomar al nitrógeno como nitrato o como amoniaco; mientras que la reducción del nitrógeno molecular, es solo competencia de procariotes, que pueden estar asociados a las plantas.

El nitrato es en general un mejor sustrato nitrogenado para las plantas, ya que puede acumularse en grandes cantidades en la vacuola, y reducirlo de acuerdo a sus necesidades, sin embargo el costo energético de su reducción es muy alto.

El amoniaco podría representar un mejor sustrato nitrogenado, ya que permanece con mas facilidad en el suelo y no reviste ningún gasto energético de transformación, sin embargo es tóxico incluso en concentraciones relativamente bajas (Givan, 1979). El amonio es, sin embargo una molécula central en el metabolismo nitrogenado, se encuentra en los tejidos en forma libre, y existe siempre la necesidad de asimilarlo o reasimilarlo para evitar su toxicidad.

Fuentes de amonio

El ion amonio proveniente del suelo es muy variable, ya que depende del estado del suelo (fertilizado o no, tipo de microflora, acidez de la lluvia etc.). En general el amonio proviene de la reducción del nitrato representa el mayor aporte de este ion al metabolismo. La reducción del nitrato, por otra parte, se lleva a cabo de una manera importante en las raices y menos activamente en las hojas. Los sistemas enzimáticos involucrados son:la nitrato reductasa, que se encuentra en el citoplasma, y que regula la entrada del nitrógeno reducido al metabolismo; y la nitrito reductasa que se encuentra en plástidos o en cloroplastos; el amonio es pues liberado aquí. El amonio, sin embargo, no parece teher barreras membranales, su transporte parece estar limitado solo al equilibrio eléctrico que debe conservarse en ambos lados de la membrana.

La fuente mas importante de amoniaco intracelular en las hojas en la mayor parte de las plantas (C-3) parece provenir de la foto-

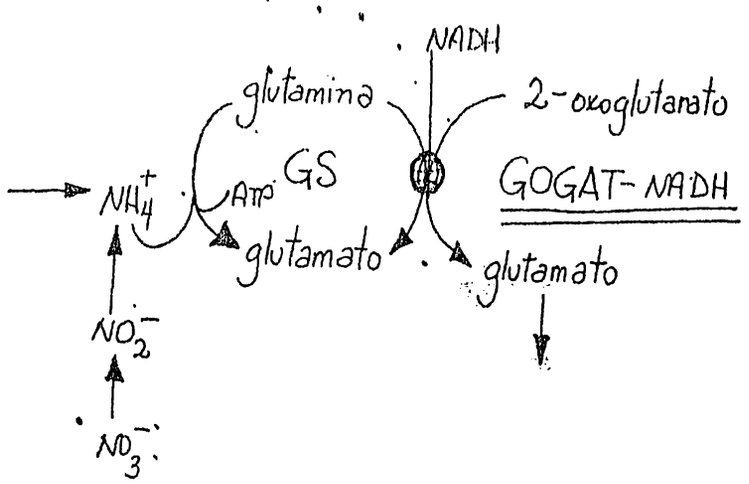
respiración en la reacción en que dos glicinas forman una serina dentro de la mitocondria (Miflin y Lea 1979) (Fig 3).En las plantas C-3 esto representa alrededor del 80% del amoniaco intracelulasr. En las plantas C-4 la fotorespiración no es detectable, o es muy baja, así que el amonio liberado por esta vía no representa una cantidad importante. En ambos tipos de plantas, durante el envejecimiento (que puede ser un proceso relativamente largo dentro de la vida de un tejido vegetal) hay tambien una importante liberación de amonio proveniente de la reacción de la fenilalanina al ácido cinámico en una reación que se lleva a cabo por la fenil alanina amonio liasa (PAL). La conversión del ácido cinámico en productos secundarios (polifenoles y derivados) representa un flujo enorme de carbono durante el envejecimiento con su concomitante liberación de amoniaco cuando el tejido no está necesariamente intoxicado por el amonio, y este debe de alguna manera reasimilarse.

Teorías para la asimilación del amonio-

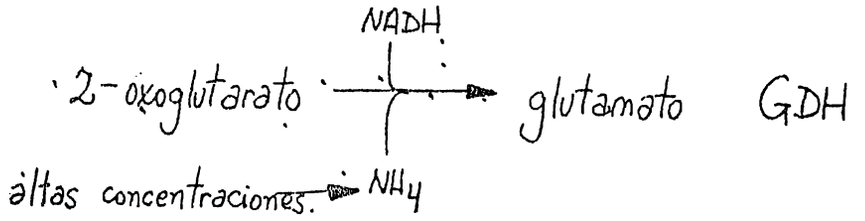
Al igual que en el resto de los organismos, antes de los 70´s se pensaba que la vía de entrada del amoniaco era a traves de la Glutamato Deshidrogenasa (GDH) y la Glutamino Sintetasa (GS) (Fig 1) Ambas enzimas proveían el glutamato y la glutamina necesarias para el resto del metabolismo. Sin embargo se encontró que la GDH tiene en todos los organismos estudiados una K_m muy elevada para el amonio; en este caso , las plantas no toleran las concentraciones del amonio de la K_m de su GDH. Esto ha llevado a pensar que la GDH tiene un papel asimilatorio del amonio solo en condiciones de toxicidad (Givan 1969)(Fig 1).

Dougall (1974), reportó por primera vez en plantas superiores, lo que ya se había descrito en microorganismos un poco antes, la existencia de una enzima capaz de producir el glutamato que requiere la GS, esta a su vez asimila el amonio con una K_m que esta dentro del rango normal de concentraciones intracelulares. Esta enzima toma a su vez la glutamina producida por la GS y tranfiere el grupo al 2-oxoglutarato usando reductores de NADH o de NADPH, produciendo así dos glutamatos. La enzima se ha llamado GOGAT (Glutamina oxoglutarato amino transferasa) o Glutamato Sintasa. A partir de entonces se ha asumido en general que esta es la vía normal de entrada del amonio al metabolismo (Miflin 1980)

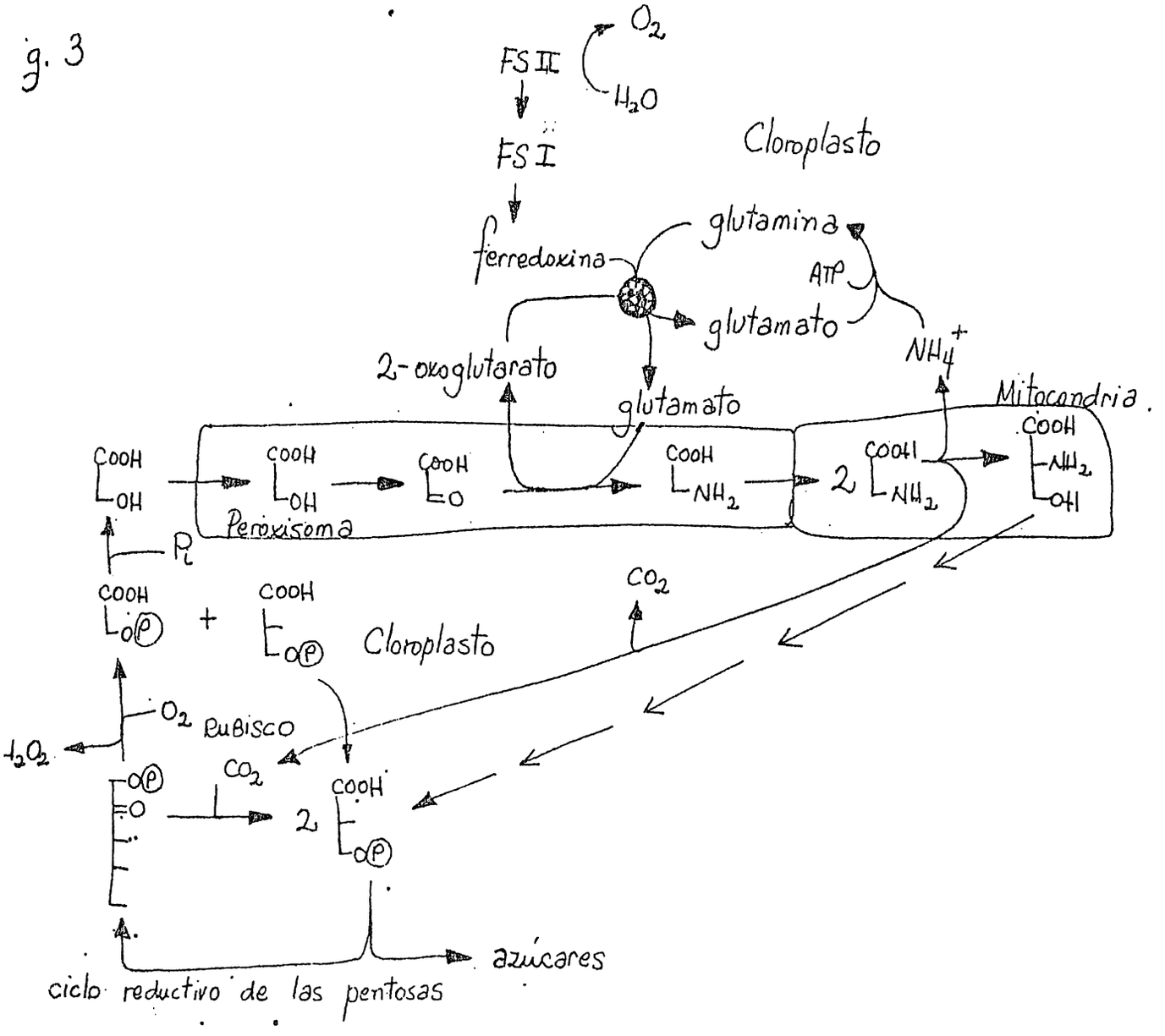
g. 1


NADH
glutamina
2-oxoglutarato
NH_4^+
ATP
GS
GOGAT-NADH
glutamato
glutamato
NO_2^-
NO_3^-

g. 2


NADH
2-oxoglutarato
glutamato
GDH
altas concentraciones
NH_4

g. 3


O_2
FS II
H_2O
FS I
Cloroplasto
ferredoxina
glutamina
ATP
glutamato
NH_4^+
2-oxoglutarato
glutamato
Mitocandria
COOH
OH
COOH
OH
COOH
O
COOH
NH_2
2
COOH
NH_2
COOH
NH_2
OH
Peroxisoma
P_i
COOH
OP
+
COOH
OP
Cloroplasto
CO_2
O_2
RuBisCO
H_2O_2
OP
O
CO_2
2
COOH
OP
azúcares
ciclo reductivo de las pentosas

(Fig 2).

El grupo de Benjamín Miflin en Inglaterra, publicó en 1975 (Miflin y Lea 1975) la existencia de un sistema enzimático que lleva a cabo la misma reacción que la GOGAT, pero esta nueva enzima funciona con ferredoxina como fuente de electrones; se ha localizado dentro de los cloroplastos. Se ha pensado que la GOGAT ferredoxina es la enzima responsable de la asimilación del amonio liberado durante la fotorespiración (Fig 3); esto se ha confirmado sobre todo por la inviabilidad de mutantes de Arabidopsis dañadas en la GOGAT-ferredoxina, en condiciones de oxígeno normal. (Ogren y Somerville 1979).

Antecedentes

Se ha desarrollado en el laboratorio un cultivo de callos de diferentes tejidos de B. ternifolia que tienen características apropiadas para llevar a cabo estudios del metablismo intermediario. El cultivo en un medio sólido (callo) y en suspensión, tiene un crecimiento rápido (en 14 días llega al estado estacionario) y es macroscópicamente y microscópicamente muy heterogéneo y blanco (Fernández y Sánchez de Jiménez, 1982). El cultivo crece bien en el medio de Murashige y Skoog (MS) (amonio y nitrato como fuentes nitrogenadas) (1962) y en medio con nitratos; en medio con amonio como única fuente nitrogenada, crece mal y eventuelmante muere o produce algunas variantes que pueden resistir el amonio; cuando se complementa con algún intermediario del ciclo de Krebs (citrato, succinato) el efecto tóxico desaparece; la glutamina no es un buen suatrato aunque no resulta ser tóxico como el amonio.

Las tres enzimas involucradas en la asimilación del amonio (GS, GDH y GOGAT) se comportan igual en los cultivos provenientes de raíz y de hoja; en el medio MS; sin embargo, con otras fuentes nitrogenadas (nitrato, amonio, glutamina, glutamato), las enzimas muestran patrones de comportamiento diferentes, que reflejan sus orígenes diferentes (Sánchez de Jiménez y Fernández 1983).

Por otra parte, los esquemas de asimilación del amonio no pueden explicar algunos datos de la literatura, como por ejemplo a) el papel de destoxificador del amonio de la GDH, no puede explicar que la GDH se encuantre en todos los tejidos, con una gran

cantidad de isoenzimas y con formas complejas de regulación (Loyola 1983), b) la GOGAT-ferredoxina se encuantra en tejidos no fotosintéticos (no fotorespiratorios) y con una buena actividad en hojas de plantas C-4 en donde la fotorespiración no es detectada o es muy baja (Matoh 1980).

A pesar de que los esquemas de asimilacion del amonio ya forman parte de un paradigma (se encuentra en textos: Encyclopedia of Plant Biochemistry Vol 5), se formula en este trabajo, una revisión de los esquemas de asimilación que se fundamenta en los datos de la literatura que no pueden ser explicados y en su falta de perspectiva dinámica. La planta es un sistema en crecimiento, desarrollo, diferenciación, ajuste al ambiente y regulación continua; así que se debería considerar la función de los sistemas enzimáticos dentro de este esquema integral, y no solamente como parte de una respuesta al origen del amonio.

Hipótesis de trabajo

Se plantea como hipótesis que, el uso alterno de las vías de asimilación del amonio en las células vegetales, no dependen exclusivamente del origen del amonio, sino que son tambien función del grado de desarrollo y la diferenciación específica de cada tejido.

Metas

1.- Medir las enzimas involucradas en el metabolismo nitrogenado en tejidos de una planta en diferentes estados de desarrollo y diferenciación.

2.- Analizar la regulación de la GOGAT-ferredoxina en el cloroplasto como modelo de una enzima ligada directamente al proceso de diferenciación de este organelo.

Diseño experimental

1.- El cultivo de tejidos de B. ternifolia ofrece un buen sistema para probar la hipótesis planteada. La diferencia en respuesta metabólica de los cultivos de callo de esta planta, mostraron en nuestro laboratorio que aún con la misma fuente nitrogenada, la actividad de la GDH, GS y GOGAT son función del origen del callo. De aquí que se eligió clonar los callos de raíz y hoja de B. ter-

nifolia para tener un sistema celular homogeneo,y de las clonas derivar cultivo de células en suspensión que permitiera hacer el estudio de las enzimas marcadoras durante el ciclo de crecimiento del cultivo.

2.- Por otro lado , el enverdecimiento del callo, es un modo de diferenciación celular sin deferenciación tisular que permite probar la hipótesis planteada; por medio de mediciones de la GOGAT-NADH y la GOGAT-ferredoxina, como marcadores para investigar si su presencia y actividad dependen de la diferenciación celular (enverdecimiento) en un ambiente en el que la fotosíntesis se lleve a cabo con alta concentración de CO_2 y por ello, la liberación de amonio fotorespiratorio sea poco probable.

3.- Por otro lado, el cloroplasto es el organelo que con mas claridad forma parte de la diferencia bioquímica entre raíz y hoja, forma parte importante del sistema del metabolismo nitrogenado, y contiene a la GOGAT-ferredoxina cuyo papel en la asimilación del amonio no esta muy clara; por esto se decidió usar como sistema experimental cloroplastos aislados de hojas jóvenes de B. ternifolia para investigar la regulación de la GOGAT-ferredoxina mas a fondo y clarificar si su papel es responder al amonio fotorespiratorio o si tiene alguna función dentro del cloroplasto como organelo especializado.

Discusión de los resultados

Artículo 1.

Alternative pathways for ammonium assimilation in Bouvardia ternifolia cell suspension cultures.

Se investigó la actividad de las enzimas involucradas en la asimilación del amonio (GDH. GS y GOGAT) así como el contenido celular de amonio en dos cultivos en suspensión de B. ternifolia, uno proveniente de raíz y otro de hoja, durante el ciclo celular. En estos experimentos se probaron diversas fuentes nitrogenadas; amonio/nitrato (MS standar), amonio, nitrato y glutamina; esta última se escogió porque en muchas plantas es el metabolito mas importante que es exportado desde la raíz a las hojas.

El cultivo proveniente de hoja y raíz se comporta de manera muy similar cuando la fuente nitrogenada es una mezcla de amonio y nitrato (Fig 1). En este caso la GDH parace ser mas importante que la GS para la asimilación del amonio, el cual se encuentra

en concentraciones relativamente altas (Fig 5). En los cultivos en que el amoniaco es la única fuente nitrogenada, éste se acumula en concentraciones aún mas altas que en la encontrada en los cultivos de la mezcla de amonio y nitrato. Se observa además un cambio relativo en las actividades de la GDH y la GOGAT: mientras la primera aumenta, la segunda es mas baja o no se detecta (Fig 2). Esto estaría de acuerdo al esquema propuesto, en el cual la GDH es la enzima responsable de la eliminación del amonio cuando éste se encuentra en concentraciones altas y en este caso la GOGAT está incluso inhibida. Cuando el nitrato es la única fuente nitrogenada, la GDH baja su actividad y la GOGAT mantiene su actividad en el tejido de hoja, mientras que en el de raíz disminuye (Fig 3). En éstos casos la actividad de la GDH es mas elevada que la de GOGAT, o la de GS; sobre todo en el caso del tejido proveniente de la raíz. Esto ya no puede explicarse completamente con el esquema original, y lo que salta a la vista es la diferencia tisular en el empleo de las diferentes vías de entrada del amonio, que reflejan probablemente la función muy particular de cada tejido. Hay que recordar que la hoja importa sobre todo aminoácidos (sobre todo glutamina y asparagina) mientras que la raíz debe reducir el nitrato y asimilar directamente el amonio.

Cuando la glutamina es la única fuente nitrogenada, se reafirma esta posición (Fig 4). En primer lugar, hay que anotar que no es ésta una buena fuente nitrogenada para ambos tejidos, ésto se observa por el menor peso fresco que se alcanza, así como la menor actividad de todas las enzimas involucradas. La GS parece estar regulada por este metabolito, ya que disminuye se actividad significativamente durante los primeros días de cultivo. Posteriormente logra recuperar su actividad; probablemente cuando la glutamina ha sido utilizada y los niveles de la poza interna han dejado de tener efecto sobre la expresión de la GS. En este caso, el tejido de raíz pierde la actividad de GOGAT hasta niveles no detectables por los métodos usados, mientras que el de hoja mantiene una buena actividad; esto podría reflejar el hecho de que la hoja en la planta, tiene a la glutamina como un metabolito normal y probablemente es metabolizado a traves de la GOGAT para producir glutamato. La vía para metabolizar la glutamina en

la raíz es seguramente otra, que puede incluir la actividad de algún tipo de glutaminasa con la liberación del amonio y su reasimilación directa, a traves de la GDH.

Por todo esto, se puede concluir que la vía de entrada del amonio en las plantas, parece depender del tipo de tejido de que se trate, ademas de el tipo de fuente nitrogenada que se le aporte al tejido.

Articulo 2.
Glutamate synthase in greening callus of Bouvardia ternifolia Schlecht.

El callo no fotosintético proveniente de hoja de B. ternifolia tiene actividad de GOGAT-NADH, pero no tiene actividad de GOGAT-ferredoxina, sin embargo el tejido enverdece (con la aplicación de benzil amino purina, ácido alfa-naftalén acético, 1.5% de sacarosa y 0.1% de CO_2, en luz)apareciendo además la actividad de GOGAT-ferredoxina, manteniendose inalterable la actividad de GOGAT-NADH (Tabla 1). Con este modelo, se trató de encontrar la influencia que tenían las diferentes fuentes nitrogenadas sobre estas dos actividades, durante el enverdecimiento de callos de B. ternifolia. Los resultados de estos experimentos mostraron que la GOGAT-NADH no está influida por el proceso de enverdecimiento. sin embargo si está influida por la fuente nitrogenada en el medio de cultivo. En el medio con nitrato como única fuente nitrogenada, la actividad de GOGAT-NADH disminuyó significativamente. La actividad de GOGAT-ferredoxina, por otro lado, apareció al mismo tiempo que la clorofila durante el proceso de enverdecimiento en los callos, aunque hay que notar que no de modo proporcional a ésta. Cuando se compara la cantidad de clorofila y la actividad de GOGAT-ferredoxina, se encuentra que no hay una correlación entre la primera y la segunda (Fig 1).

Para conocer un poco mas la función posible de cada una de etas enzimas, se estimó necesario conocer primero su localización en los tejidos. Para ello se usaron dos técnicas; la centrifugación diferencial, y un gradiente continuo-discontinuo de sacarosa. En los experimentos de centrifugación diferencial, se encontró actividad de GOGAT-NADH tanto en los callos verdes como

en los blancos en las fracciones de 2 500 g o en la de 5 000 g (plástidos y cloroplasto repectivamente) y en el citoplasma (sobrenadante de 20 000 g), mientras que en el tejido de hoja se encontró en los cloroplastos pero no en el citoplasma (Tabla 2). La contaminación de los organelos entre las fracciones y la ruptura de estos fué muy baja según indica la distribución de las enzimas marcadoras usadas en estos experimentos. En los experimentos de gradientes de sacarosa, se logró una buena resolución en la separación de los organelos según lo indican los mismos marcadores usados (Fig 2). La distribución de ambas GOGAT fué la misma que en el caso de fraccionamiento: la GOGAT-ferredoxina se encuentra solamente en los cloroplastos, mientras que la GOGAT-NADH se encuentra distribuida tanto en plástidos como en citoplasma en los dos cultivos usados: fotosintéticos y no fotosintético. Por otra parte, en la hoja las dos enzimas se localizaron exclusivamente en el cloroplasto. Hay que hacer notar que el callo verde tiene una baja concentración de clorofila y una baja actividad de GOGAT-ferredoxina, por esto, se considera que se trata de un tejido intermedio entre el tejido no fotosintético (callo blanco) y uno verdaderamente autótrofo como la hoja; esto explicaría que en el callo verde, la GOGAT-NADH se encuentra dividida en el citoplasma y en los plástidos, mientras que en un tejido completamente autótrofo esta actividad ya no se detecta en el citoplama. Esta distribución parece sugerir que existen en estos tejidos dos isoenzimas de GOGAT-NADH: una citoplásmica y otra particulada. Esta sugerencia se ve apoyada además por los experimentos realizados con antibióticos (Tabla 3), los cuales muestran que las dos actividades de GOGAT-NADH se ven afectados por los antibióticos de manera diferente.

De estos resultados se puede concluir que la función metabólica de ambos tipos de GOGAT, debe ser diferente, la GOGAT-NADH está ligada con la fuente nitrogenada, y una posible isoenzima(la citoplasmática) está restringida solamente a cierto tipo de tejidos no fotosintéticos. Finalmente la GOGAT-ferredoxina parece estar ligada a la fotosíntesis, por su aparición durante el enverdecimiento del tejido y por su localización intracelular.

Artículo 3.

Regulation of ferredoxin-GOGAT in isolated chloroplasts.

Se hicieron estudios de regulación en cloroplastos aislados de B. ternifolia con objeto de entender el funcionamiento y la regulación de la enzima GOGAT-ferredoxina. Los cloroplastos de B. ternifolia obtenidos mostraron una buena estabilidad ya que se mentienen metabolicamente activos en la síntesis de proteínas (Fig 5) y en el transporte y la transformación de metabolitos en presencia de luz (Fig 3 y 4) durante el período de experimentación (3 horas). Por otra parte, a las tres horas de incubación en luz la actividad de GOGAT-ferredoxina se mantiene sin cambio alguno, sin embargo en oscuridad se pierde la mitad de la actividad en este tiempo (Fig 1). Este fenómeno se usó como modelo para tratar de encontrar el mecanismo de regulación de esta enzima.

En primera lugar, los cloroplastos incubados en oscuridad pueden recuperar parte de la actividad de GOGAT-ferredoxina si se les incuba en luz de nuevo (Fig 1). Esto indica una posible regulación de la enzima por algún fenómeno directamente relacionado con la fotosíntesis. Se trató de dilucidar si los cambios en el pH y en las concentraciones de Mg^{++} eran los responsables de este fenómeno, sin embargo ninguno de estos elementos parece afectar la actividad de la enzima al romper los cloroplastos (Fig 2). Un fenómeno muy estudiado, ha sido la regulación de la actividad de las enzimas cloroplásticas por medio de intermediarios que reducen puentes de disulfuro y que responden al sistema de transporte de electrones. Se trataron dos de estos metabolitos (ditiotreitol y mercaptoetanol) pero no parecieron influir en la recuperación de la actividad de la enzima (Tabla 1). Por último se trataron algunos reductores directamente, tales como NADH NADPH y ferredoxina, pero ninguno de estos influyó en la recuperaciónde la actividad.

Se pensó en una regulación por sustratos; así que se incubaron los cloroplastos con los metabolitos de la GOGAT (glutamina y 2-oxoglutarato) o del ciclo completo GS/GOGAT (Glutamina, 2-oxoglutarato y amonio). Los resultados indicaron que la producción de glutamato por los cloroplastos iluminados dependió de la mezcla

de estos metabolitos, la mezcla completa resultó mucho mas efectiva y la eficiencia aumentó en todos los casos al añadir malato al sistema. Por otra parte, cuando se probó este sistema en la oscuridad, se observó que la producción de glutamato fué muy pequena, lo que indica la dependencia de la reacción a la luz. Sin embargo la presencia de los metabolitos no impidió que en oscuridad se perdiera igualmente la actividad de la GOGAT-ferredoxina, lo que indica que no son los metabolitos intracloroplásticos los que regulan la actividad de la enzima (Fig 3).

Por otra parte, experimentos en presencia de luz con la adición de DCMU (inhibidor especifico muy efectivo de la fotosíntesis) mostraron una pérdida significativa de la actividad de la enzima a las 3 horas de incubación (aunque de menor intensidad que con la incubación en oscuridad) lo cual indica una dependencia de la enzima de la función fotosintética, aunque la fotosíntesis es solo una parte de la explicación de la regulación de esta enzima, pero no podría explicarla totalmente (Fig 4).

Se investigó la síntesis de proteínas como fuente de regulación de esta actividad. La síntesis de proteínas puede inhibirse completamente con la adición de 50 μg de cloramfenicol por ml a los cloroplastos (medida como inhibición de la incorporación de aminoácidos C^{14} a proteínas). La inhibición de la síntesis de proteínas en los cloroplastos aislados también hace disminuir la actividad de GOGAT-ferredoxina en forma muy dramática. La pérdida de actividad sigue una cinética de primer orden, así que se calculó la vida media de la proteína involucrada en esta actividad, y se encontró de 1 hora con 43 minutos (Fig 5). Esta es una vida media bastante pequeña para el promedio de la vida media de otras proteínas, aunque no muy pequeña si se compara con la vida media de la nitrato reductasa (3 horas en las puntas de las raíces y 2 horas en partes maduras de la raíz) reportada en 1972 Por Ann Oaks en Canadá. Estos resultados no permiten afirmar sin embargo que ésta sea la vida media de la GOGAT-ferredoxina, solo se puede decir que hay una proteína que tiene un recambio rápido y que influye en la actividad de la enzima. Por esto, se purificó la enzima (por aislamiento de cloroplastos, precipitación con acetona, diálisis y elución por un gradiente salino de una columna de DEAE-celulosa) 350 veces. Se obtuvieron anticuerpos de

ésta y se determinó en el antisuero un título de 1:15. Muestras de cloroplastos, se incubaron en luz, oscuridad o con cloramfenicol a diferentes tiempos, se tomaron 5 unidades de actividad (con un aumento en el volumen de ensayo) y se añadieron los anticuerpos que precipitarían 5 unidades de actividad al tiempo cero. Si la actividad fuera idéntica a la proteína presente, se esperaría que los anticuerpos precipitarían siempre a las 5 unidades de actividad, si la pérdida de actividad fuera inactivación de la enzima, habría enzima inactiva sin ser precipitada y con esto se detectaría actividad remanente. Esto último fué lo que sucedió, los cloroplastos incubados con cloramfenicol, mostraron actividad después de una hora de incubación, a las dos horas se encontró una mayor actividad; los cloroplastos incubados en oscuridad, mostraron actividad después de dos horas y ésta aumentó hasta las tres horas, por último, algo que no se esperaba, es que incluso los cloroplastos incubados en luz, mostraron actividad a las tres horas (Figura 7). Esto indica que la pérdida de actvidad en oscuridad y por la inhibición de la síntesis de proteínas se debe a la pérdida de actividad de la enzima por modificación, o por degradación parcial de la enzima manteniendo los determinantes antigénicos. Se puede argumentar en la misma dirección, que existe una proteína de recambio rápido que influye en la actividad de la GOGAT-ferredoxina. Según este modelo de regulación, puede pensarse que la GOGAT-ferredoxina funciona en la medida que hay fotosíntesis luminosa y que su actividad está regulada por ésta y por la síntesis proteica. Esto sugeriría que la enzima esta funcionante como parte de un sistema energético en el que participa la fotosíntesis. Dentro de este modelo se podría pensar que por intermediación d la GOHAT-ferredoxina, los reductores generados en el cloroplasto serían exportados como glutamato al citoplasma y/o la mitocondria donde se donarían los reductores al NAD^+ por la acción de la GDH.

Conclusiones

1.- Los resultados experimentales apoyan la conclusión de que la hipótesis presentada es verdadera. El uso alterno de las vías de asimilación de amonio mostraron ser tanto dependientes del origen de la fuente de nitrógeno que se aportó al tejido como de el estado de diferenciación y de su desarrollo.

2.- En los dos primeros casos, la respuesta enzimática fué función del origen del tejido (proveniente de raiz y de hoja) y del proceso de enverdecimiento, pero también hay una respuesta a la fuente externa de nitrógeno.

Esta respuesta no es simplemente al origen del amonio, sino aún más es un complejo ya que por ejemplo, la respuesta del tejido cuando solo amonio es la fuente nitrogenada es muy diferente a la del amonio con citrato o nitrato, ya que en estos dos últimos casos el amonio no tiene ningún efecto tóxico.

3.- El nitrato por otra parte esta ligado a la vía GOGAT-NADH, ya que influye en la respuesta de esta enzima en el cultivo en suspensión (tejido de raíz) y en el cultivo de la hoja durante el enverdecimitno.

4.- La regulación de la GOGAT-ferredoxina haría pensar que la respuesta de esta enzima es función de la actividad fotosintética del tejido. Sin embargo, falta aún investigar la relación de esta función con el amonio fotorespiratorio.

En resúmen, la vía de asimilación de amonio en las plantas depende tanto del origen de éste compuesto, como del tejido específico del que se trate.

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VERSIDAD NACIONAL
AVTONOMA

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Universitat Innsbruck

A-6020 Innsbruck, Austria

Dear Professor:

We are writing you to acknowledge your letter concerning our manuscrupt 2703. The main objetion to this manuscript, as you indicate in your letter, is the possible overlapping of information between this manuscript and our previous paper submitted, and already published in Planta. As you can see in the reprint we are enclosing, the Planta article deals with the measurement of several enzyme activities as indicators of the dedifferentiation state of B. ternifòlia callus tissue at one determined stage of development. Both calli showed the same biochemical behaviour in MS medium (nitrate and ammonium as nitrogen sources), but they did not respond in the same way when grown in other nitrogen sources; so, it was concluded that "calli originated from different B. ternifòlia tissue do not have the same biochemical dediffenentiated state".

On the other hand, the manoscript we are submitting for publication in Z. Pflanzenphysiologie (2703) deals with the regulatory mechanism of the nitrogen metabolism. The main goals are to elucidate the role of GDH-GS and GS/GOGAT pathways in ammonium assimilation. Although GS/GOGAT has been repeatedly shown to be the physiological pathway to assimilate ammonium, the role of GDH in this process is not clear. The main contribution of this manuscript, we think, is to show that GDH is a highly responsive enzyme to different nitrogen sources as well as to different stages of the cell growth, this suggests that the role of GDH in normal ammonium assimilation cannot be ruled out. The only conection between the two papers, is that both show that cell cultures originated from root and leaf, do not have the same biochemical behaviour.

Regarding the other referee comments:
1) The measurements of ferredoxin-GOGAT activity have been carried out with methyl viologen as well as with ferredoxin.

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We have been working specifically with this enzyme in green callus and leaves using both assay conditions, with similar results. A statement clarifying this point has been included in the manuscript.
2) Regarding the non-published auto-citations; one is the Planta article, already published (the reprint is enclosed), and the other two have been deleted from the new manuscript.
3) The experimental repetitions refered in the old manuscript were carried out with different cell lines (clones) obtained from different explants. To clarify this fact, we have pointed out this in the new manuscript.
4) The explanation for the figures as well as the titles in the references have been included.
5) The indication of the origin of the cell line has been stated in Table 1.
6) The English text has been reviewed by an English speaking person, and has been improved.

Taking into account the above points, we are sending back to you the reviewed manuscript for re-consideration for publication in Zeitschrift fÜr Pflanzenphysiologie.

Thanking in advance for your attention:

Sincerely yours

_____

Ezequiel Murillo

ALTERNATIVE PATHWAYS FOR AMMONIUM ASSIMILATION IN *Bouvardia ternifolia* CELL SUSPENSION CULTURES

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Summary

Cell suspension cultures of young leaves(L) and roots (R) of Bouvardia ternifolia were cloned and their growth was followed, measuring cell volume and fresh weight. Both cultures were depleted of nitrogen and subcultured in different nitrogen sources. The enzymes for ammonium assimilation and the ammonium pools were measured during the growth cycle.

Results indicate taht the two ammonium assimilation pathways, the GS/GOGAT, and the GDH-GS, are functional in both cultures. The pathway used is dependent upon the tissue and the nitrogen source in the medium. GDH seems to have an anabolic role on root cultures and is regulated by the internal concentration of ammonium.

Key words: Bouvardia ternifolia, ammonium assimilation, cell suspension culture, nitrogen metabolism.

Introduction

Ammonium can be assimilated at different points on the intermediate metabolism (Miflin, 1977). There are, however, two main streams in which most of the ammonium is incorporated into some central metabolites (Miflin, 1980): the Glutamine Synthetase/Glutamate Synthase (GS/GOGAT) cycle and the Glutamate Dehydrogenase-Glutamine Synthetase (GDH-GS) pathway.

The entrance of ammonium via GDH has been questioned since GDH has a very high K_m for ammonium that is above the normal cellular concentrations. This has led to the conclusion that GDH has either a detoxifying role or that it works mainly in the deaminative direction (Givan, 1979; Miflin, 1977). On the other hand, GS has a lower K_m for ammonium, and glutamate production has been found to be coupled with GOGAT in most plant tissues (Yoneyama and Kumazawa, 1974, 1975; Lea and Miflin, 1974; Steward and Rhodes, 1976; Arima and Kumazawa, 1977). Moreover, the amount of ATP needed by the GS reaction makes glutamate formation through this cycle energetically more favorable.

Recent data however suggest an assimilation role for GDH in some plant tissues (Pahlich, 1975; Oaks, personal communication), on the basis of 1) the abundancy of GDH in most plant tissues (Lees and Dennis, 1981), b) its distribution as different isoenzymes (Barash et al., 1973; Nauen and Hartman, 1980), and c) its low K_m value for ammonium in

pre-steady state conditions (Pahlich and Gerlitz, 1980). Therefore, the question of how different tissues regulate the use of the two alternative pathways for ammonium assimilation depending on their physiological stage and availability of nitrogen compounds remains open.

Previous work in our laboratory has shown that callus from B. ternifolia leaves and roots retain some of the regulatory mechanism of the nitrogen metabolic paths from the original tissue (Sánchez de Jiménez and Fernández, 1983) and we thought that cell lines cultured in vitro could be good systems to examine the specific role of the alternative pathways in different tissues, at different stages of growth and with different nitrogen sources. To avoid biochemical heterogeneity of the cell population we used cloned cell lines.

Materials and Methods

Cell suspension cultures.

Cell suspension cultures were obtained from callus previously generated from young roots and leaves (Fernández and Sánchez de Jiménez, 1982). Suspension cultures were obtained disgregating 5 g of callus in 50 ml of Murashige and Skoog (1962) (MS) medium supplemented with 1 mg/ml of 2,4-D and 0.005 mg/l of kinetin. The cultures were incubated at 26°C on a horizontal shaker at 160 rpm under light (20 Wm^{-2}). Further subculturing was carried out transferring 5 ml ($5x10^5$ cells/ml) of this inoculum to 25 ml of fresh medium in 125 ml flasks.

Growth curves.

Cells were suspended in fresh MS medium without nitrogen for 48 hours under the conditions described above. After that period, $5x10^5$ cells were inoculated in 125 ml flasks containing MS supplemented with either 20 mM KNO_3, 10 mM NH_4Cl or 5 mM glutamine and cultured for 15 days. Two samples from each flask were taken every two days for cell volume and enzymatic assay determinations. The ammonium and glutamine media were supplemented with 20 mM KCl and the ammonium medium was also supplemented with 10 mM citrate (pH 7).

Packed cell volume (PCV).

PCV was obtained centrifuging a 10 ml sample of the growing cells in a graduated conic tube at 3000 xg for 20 minutes and recording the cell volume.

Fresh weight.

25 ml of cell culture were vacuum filtered on filter paper of known weight. Fresh weight was determined by the weight difference in duplicated samples.

Cell plating.

The method described by Weber and Lark (1979) was followed: B. ternifolia suspension cultures from root tissues were used as feeder cells in the system. Three selection cycles were carried out.

Enzyme activities.

Enzyme activities in cell culture homogenates were mea-

sured as follows: NADH-GOGAT was extracted in 0.05 M potassium phosphate buffer (pH 7.5) containing 10 mM EDTA and 1% β-mercaptoethanol using a Polytron homogenizer at full speed (20 000 rpm) for 60 seconds. After filtration through 8 layers of gauze the extract was centrifuged at 10 000 xg for 15 minutes and GOGAT activity was measured immediatly in the supernatant. The assay medium contained 0.6 ml of 50 mM 2-oxoglutarate, 0.5 ml of 50 mM glutamine (both neutralized to pH 7 with KOH), 0.2 ml of 0.05 M phosphate buffer (potassium salt) (pH 7.5) and 0.5 ml of crude homogenate. The reaction was initiated by adding 0.2 ml of 10 mM NADH and the activity measured following the absorbance at 340 nm.

Ferredoxin-GOGAT. Green callus or young leaves were homogenized in phosphate buffer (0.05 M potassium salt, pH 7.5, 10 mM EDTA and 0.5% β-mercaptoethanol) using a Polytron homogenizer at full speed (20 000 rpm) for 30 seconds. This extract was filtered through 8 layers of gauze, centrifuged at 10 000 xg for 20 minutes and the supernatant used as the source of crude enzyme. The activity was measured according a Matoh (1979) using methyl viologen or ferredoxin as the electron donor. Glutamate was determined by the ninhydrin method (Moore and Stein, 1948).

GDH (aminative). The enzyme was extracted and measured as previously reported (Sánchez de Jiménez and Fernández, 1983) with the exception that instead of EDTA, 2 mMoles of

$MgCl_2$ were added.

GS. The crude extract was obtained homogenizing the tissue in 0.05 M Tris-HCl, 1 mM EDTA and β-mercaptoethanol following the same procedure used for GOGAT-NADH. The enzyme activity was measured according to Elliott (1953).

Protein. Protein concentrations were determined according to the Biuret method (Gornall et al. 1949) using bovine albumin as standard.

Ammonium. Cells were centrifuged and washed three times in MS medium without any nitrogen and homogenized in potassium phosphate buffer (pH 7) 0.01 M (4°C). The homogenized cells were filtered through 8 layers of gauze and centrifuged at 10 000 xg for 15 minutes. Ammonium was determined in this supernatant by the Nessler method (Streuli and Averell, 1970).

Results

Fine cell suspension cultures were obtained from B. ternifolia leaf (L) or root (R) callus. The cells of both cultures grew fast increasing their fresh weight almost 20 times every 15 days. To avoid somatic variability clones were obtained for each culture. Cells were plated following the method of Weber and Lark (1979) and colonies were recovered. Colonies were allowed to grow to obtain enough tissue to carry out a new plating cycle. This procedure was repeated three times to assure that the selected colonies were of a single cell origin and therefore genetically homogenous tis-

sue. Clones of R and L calli were used as the inoculum for cell suspension cultures. The growth curves of these cultures as well as the enzyme values were highly reproducible when single cell cultures were used shortly after their isolation. Noncloned cultures showed the same general biochemical patterns though with larger dispersion. The results presented below were repeated with at least two different clones obtained independantly.

Both cultures showed a normal exponential growth curve in MS medium measured by packed cell volume and by fresh weight (Fig. 1a and 1b) though R cultures, seem to have a slightly longer lag period than the L ones. The enzymes for ammonium assimilation (GS, Glutamine syntethase; GDH, Glutamate dehydrogenase; and GOGAT, Glutamate synthase) were measured in homogenates of both cultures during differents stages of growth. The three enzyme activities showed similar but not identical patterns for both cell cultures. It is important to point out that GDH from roots rises and reaches its highest level at earlier stages of growth than the leaf enzyme. The GS activity on the other hand rises along with the growth curve and reaches a plateau before the stationary phase (Fig. 1a and 1b). NADH-GOGAT activity has a similar pattern in the two cultures, but it is slightly higher in leaf cells. Ferredoxin-GOGAT was not detected in either culture at any stage of growth.

When cells were grown in a medium where ammonium was the only nitrogen source (supplemented with citrate) (Fig. 2a and 2b), the increase in cell volume and in fresh weight was about one third that of the observed in MS medium (control). The initial lag period was the same in both, R and L cultures. GS activity showed very similar values to those of the control (MS) in both tissues while GDH showed significant differences: in leaf culture its maximum level was reached earlier than in the control, yet the R culture did not reached its peak until the seventh day. The activity was, however, significantly higher in this medium than in the control. GOGAT activity showed a slight decrease at the early stages of growth in L cultures (Fig. 2a and 2b). A dramatic decrease of this activity was observed at the early stages of growth in the R cultures, and from the sixth day of incubation and thereafter, no detectable activity was found.

Cells growing in a medium with nitrate as the only source of nitrogen showed very similar curves to the ones growing in MS (Fig. 3a and 3b). The enzyme activities, however, showed considerable differences with the exception of GS which maintained the same level and pattern of the control. GDH activity showed a flat curve in both cultures with a tendency to increase at later stages in R cells. In L cells, GOGAT activity increased significantly, following a parallel pattern with the GS activity during part of the growth curve (Fig. 3a) but in R cells, a decrease in activity was observ-

ed at the early stages of the growth curve, and the level remained low for the rest of the experimental period (Fig. 3b).

Finally, when cells were cultured in a medium with glutamine as the only nitrogen source, many changes were observed (Fig. 4a and 4b). Growth was significantly slower in both cell types, and reached its stationary phase when the inoculum had increased to only about 8 times its original cell volume. GS activity declined at early stages in both cultures (Fig. 4a and 4b), and recovered slowly towards the end of the incubation period. GDH activity showed a mild increase in R cultures, where it reached a peak on the eleventh day, but increased very slowly in L cultures. GOGAT activity has a significant increase after the fourth day of culture in L cells but in R cells showed a dramatic decrease to non-detectable levels on the sixth day.

Cross-experiments were carried out to rule out the possibility of GOGAT inactivation by inhibitory factors liberated during tissue homogenization. L cells from different stages of growth in nitrate medium were mixed and homogenized with R cultures from the same age, grown either in glutamine or ammonium and were processed to measure GOGAT activity. The results shown in Table 1 gave the expected additive values, indicating the absence of inhibitors or activators of enzyme activity in the extracts.

Ammonium pools were measured during the growth stages. This ion reached higher concentrations inside the cells when they were grown in a medium supplemented with ammonium and citrate than when grown in ammonium with nitrate (Fig. 5). However, the maximum level was reached later in the first medium. Glutamine produced higher concentrations of ammonium than did nitrate, but both levels were well under those observed when ammonium was the nitrogen source. In the root cell line, the curves followed the same pattern.

Discussion

Our results show that the two ammonium assimilating enzymes: GDH and GS, and both glutamate producing enzymes: GDH and GOGAT, are present and active along most of the growth curve in different nitrogen sources and different tissues. The regulation of these enzymes is under control of the nitrogen compounds used, and varies from one tissue to another. GDH seems to be associated to the anabolic pathways rather than with catabolic functions, since its increase preceds the log phase of growth, this was more noticeable in R cells. This finding has also been observed in other plant tissues (Kanamori et al. 1972). High levels of GDH activity in all cases seem to follow the intracellular ammonium accumulation (Fig. 2a, 2b and 5) suggesting a regulatory role for this metabolite. There are several reports on other tissues where ammonium induces GDH activity (Kanamori et al. 1972; Borash et al. 1975; Davis and Texei-

ra, 1975).

GS does not seem to be the central enzyme in the regulation of ammonium in B. ternifolia cell cultures, since it keeps practically the same pattern of activity in both tissues and in all media tested. This might reflect a constant need for glutamine in the tissue to support the synthesis of nitrogenated bases needed for fast cell division. If this is so, glutamine and not ammonium would regulate the activity of this enzyme. This is in agreement with the results obtained with glutamine as the only source of nitrogen in the medium (Fig. 4a and 4b). In this case, GS diminishes its activity at earlier stages of the curve and latter on recovers slowly to its original value, presumably when glutamine has been partially metabolized. This kind of regulation has been reported before for lupin embryonic axes (Ratajczak et al. 1981) and roots of Datura stramonium (Probyn and Shargool, 1979).

GOGAT seems to have a relevant role in L cells to produce glutamate when glutamine is in the medium or when ammonium is derived from nitrate. GOGAT reaches its highest level of activity under these conditions (Fig. 4a and 4b). It is interesting to note that in B. ternifolia callus NADH-GOGAT is partially compartmentalized inside plastids (Murillo and Sánchez de Jiménez, 1983) where nitrate-ammonium conversion takes place. On the other hand, this does not seem to be the case for R cells where GOGAT is not active for most of the growth period when growing with nitrate or

glutamine. The presence of high glutamine concentration in this tissue seems to switch the entrance of ammonium through the GDH path rather than the GS/GOGAT cycle, as suggested previously for Lemna cells (Steward and Rhodes, 1976). This behaviour might reflect either a more sensitive GOGAT regulation in leaf tissue, and/or a different path for glutamine metabolization, e.g. glutamine deamination followed by ammonium reassimilation through GDH. Accordingly, R cells growing in a medium containing glutamine show an increase in GDH activity (Fig. 4) that might represent a response to an increase in ammonium produced by glutaminase activity.

We can conclude that normal B. ternifolia cells assimilate ammonium through both GDH and GS/GOGAT enzyme systems, and that the preferential use of either pathway depends on the type of tissue, the source of nitrogen and the phase of the growth curve.

Acknowledgments.

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TABLE 1. CROSS TEST OF GOGAT ACTIVITY FROM TISSUES WITH AND WITHOUT ENZYME ACTIVITY

Days of growth	Total activity of cells derived from leaf, grown on NO_3^- medium (2 g of tissue)	Total activity of cells derived from root, grown on gln medium (2 g of tissue)	Total activity of cell mixture (leaf and root cells in NO_3^- and gln medium, 2 g each tissue)
6	26.4	0.0	27.2
9	31.1	0.0	29.6
12	27.0	0.0	28.8

Two grams of fresh tissue were homogeneized and the GOGAT activity (nMole NADH/min) measured. In the mixture two grams from each tissue was taken and homogeneized together. The results are the mean of three independent measurements.

Acknowledgments:

This research was supported in part by grant No. PCCBNAL-790076 from Conacyt (Consejo Nacional de Ciencia y Tecnologia, Mexico).

Legends to figures

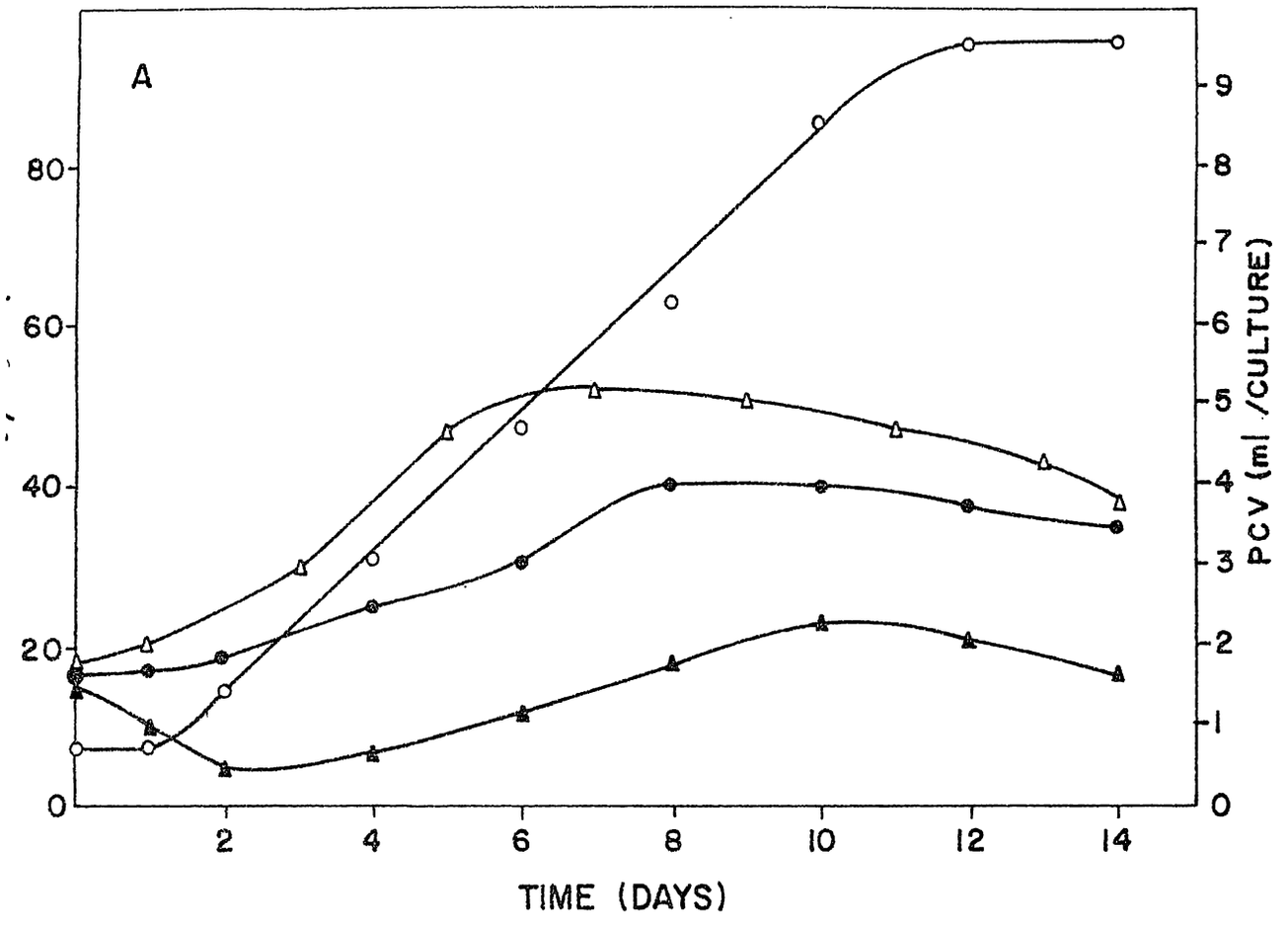
Fig. 1A & 1B. Changes in cell volume (○), GDH activity (in nMoles of NADH oxidized/min/mg of proteins)(△), GS activity (in nMoles of glu-OH-NH_2 formed/min/mg of proteins)(●) and GOGAT (in nMoles of NADH oxidized/min/mg of proteins)(▲), during the growth curve of B. ternifolia cell culture derived from leaf (A) and root (B). The culture was grown in MS medium.

Fig 2A & 2B. Changes in cell volume (○), GDH activity (in nMoles of NADH oxidized/min/mg of proteins)(△), GS activity (in nMoles of glu-OH-NH_2 formed/min/mg of proteins)(●) and GOGAT (in nMoles of NADH oxidized/min/mg of proteins)(▲), during the growth curve of B. ternifolia cell culture derived from leaf (A) and root (B). The culture was grown in MS medium with ammonium (10 mM) as the only nitrogen source, added with citrate (10 mM).

Fig 3A & 3B. Changes in cell volume (○), GDH activity (in nMoles of NADH oxidized/min/mg of proteins)(△), GS activity (in nMoles of glu-OH-NH_2 formed/min/mg of proteins)(●) and GOGAT (in nMoles od NADH oxidized/min/mg of proteins)(▲), during the growth curve of B. ternifolia cell culture derived from leaf (A) and root (B). The culture was frown in MS medium with potassium nitrate (20 mM) as the only nitrogen source.

Fig 4A & 4B. Changes in cell volume (○), GDH activity (in nMoles of NADH oxidized/min/mg of proteins)(△), GS activity (in nMoles of glu-OH-NH_2 formed/min/mg of proteins)(●) and GOGAT (in nMoles of NADH oxidized/min/mg of proteins)(▲), during the growth curve of B. ternifolia cell culture derived from leaf (A) and root (B). The culture was grown on MS medium with glutamine (5mM) as the only nitrogen source.

Fig 5. Ammonium changes during the growth curve of leaf derived cells in MS medium with different nitrogen sources: standard MS medium (nitrate plus ammonium)(●), MS with ammonium plus citrate (○), MS with glutamine (▲) and MS with KNO_3 (△).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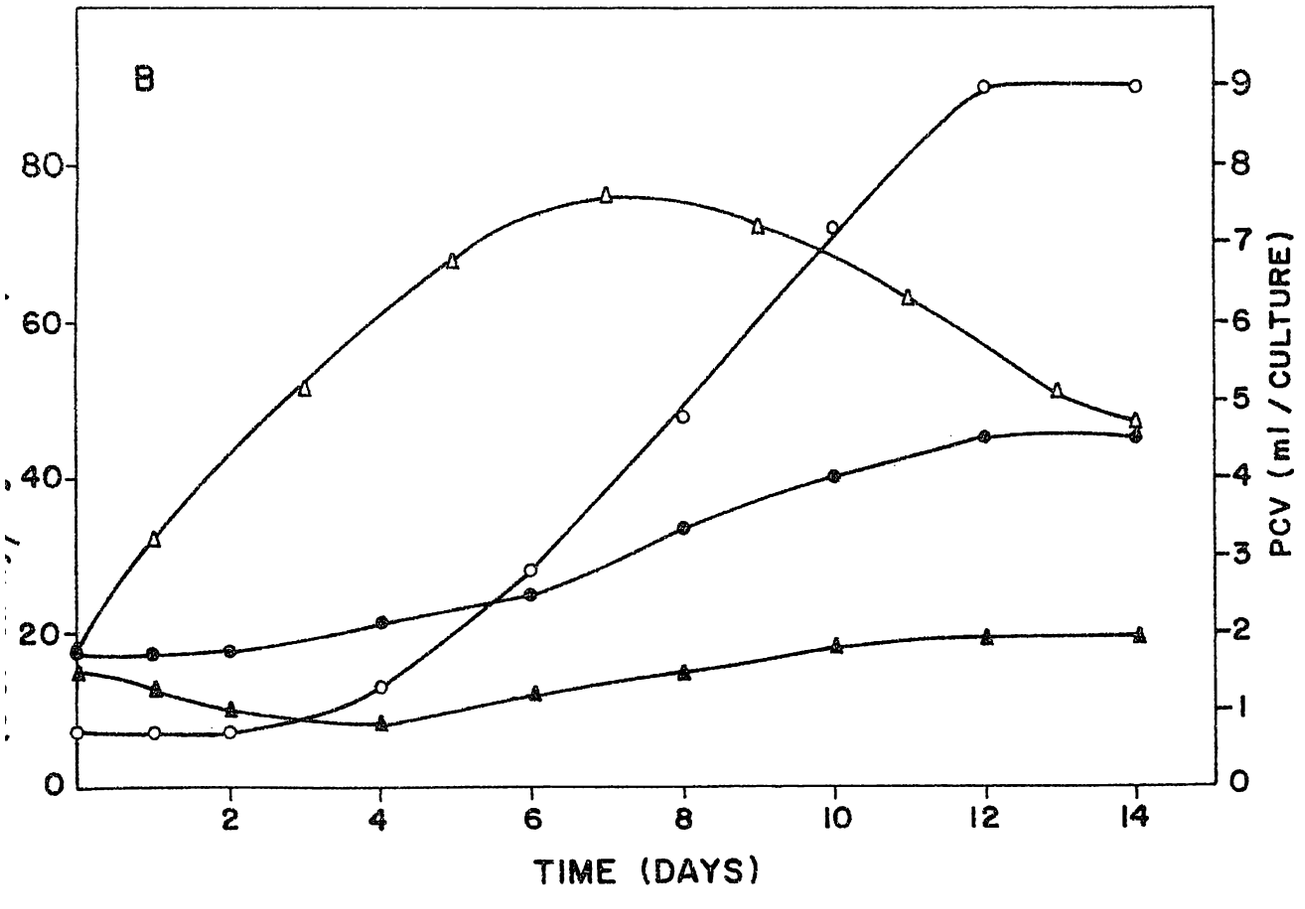
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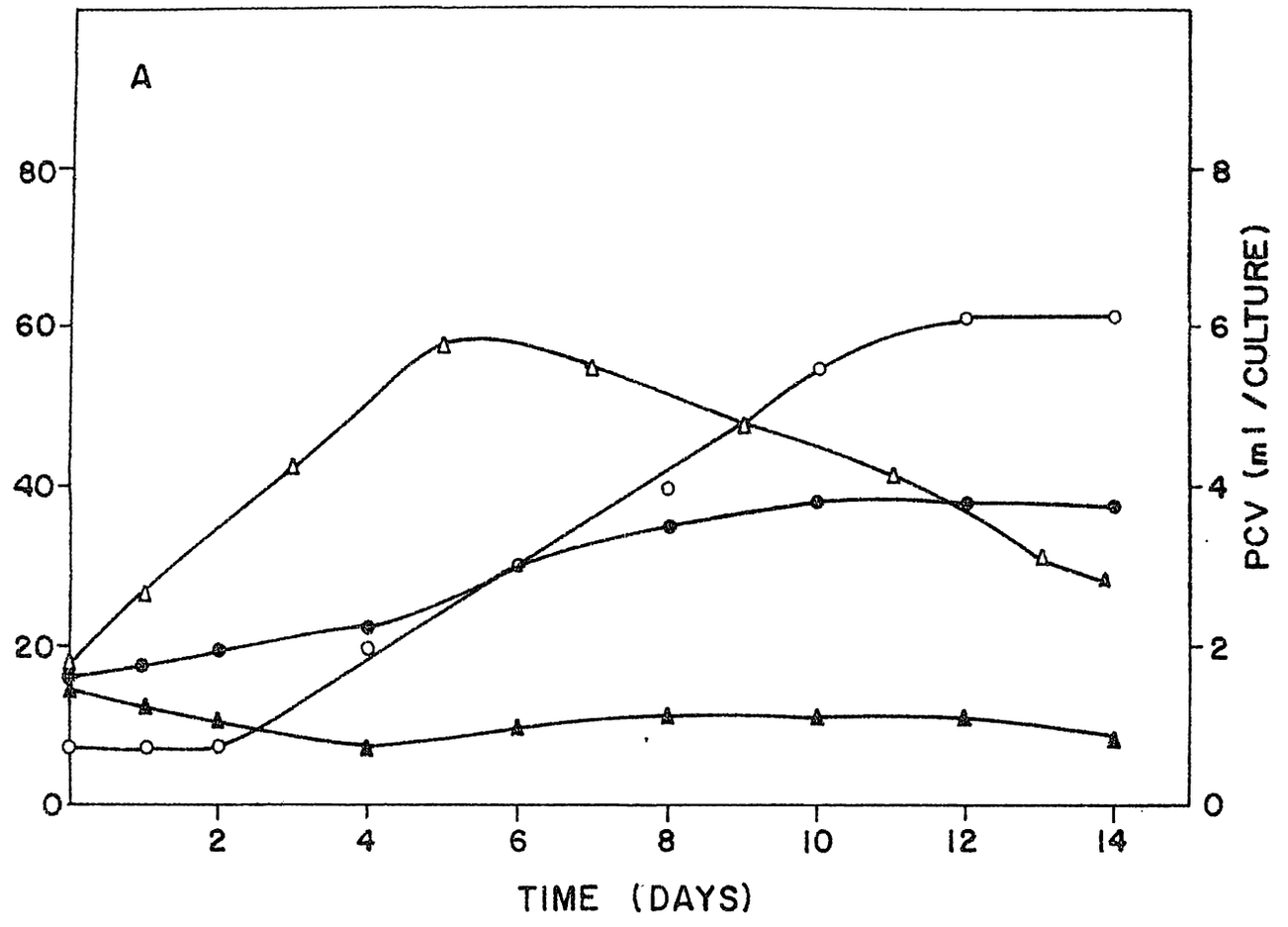
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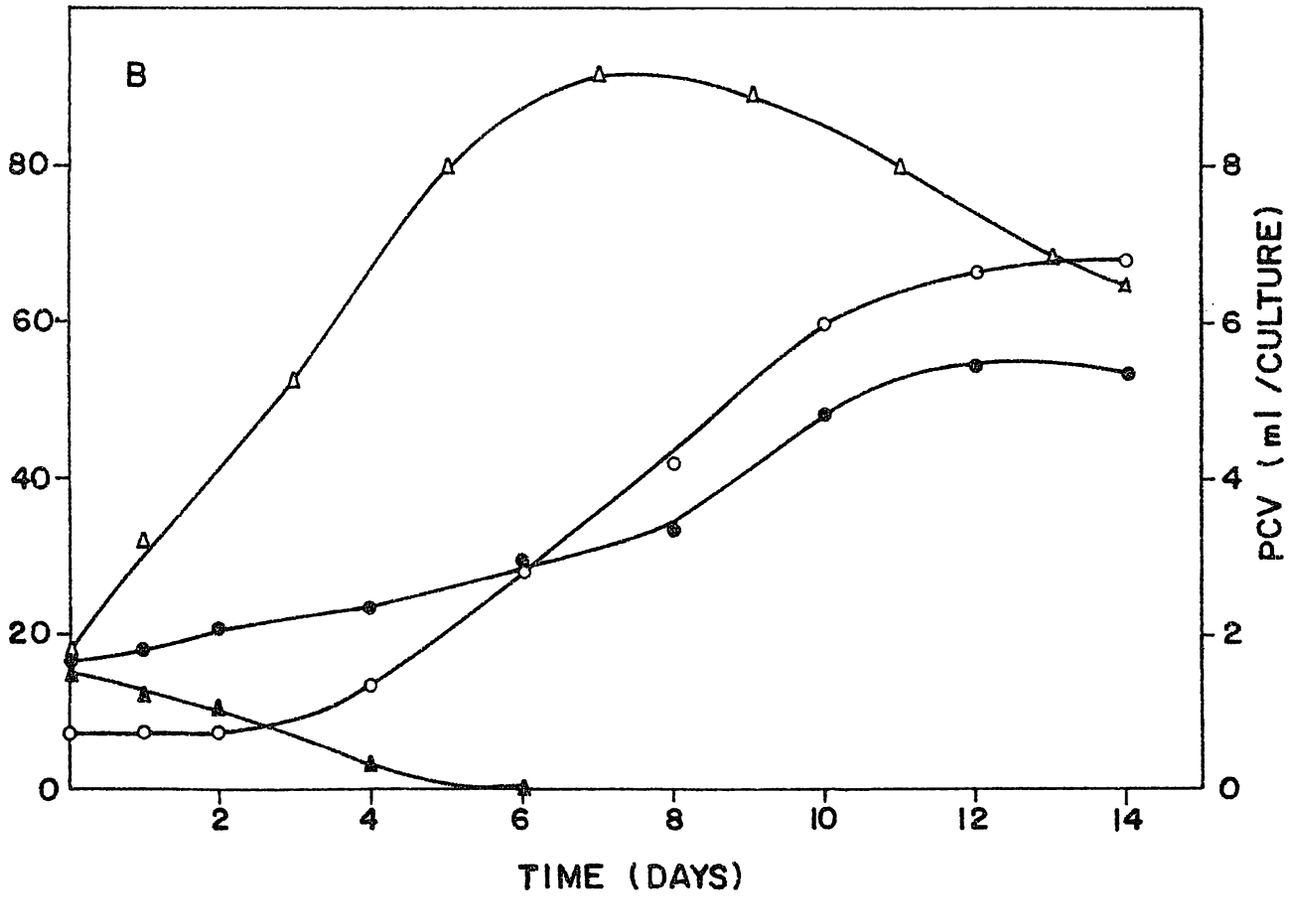
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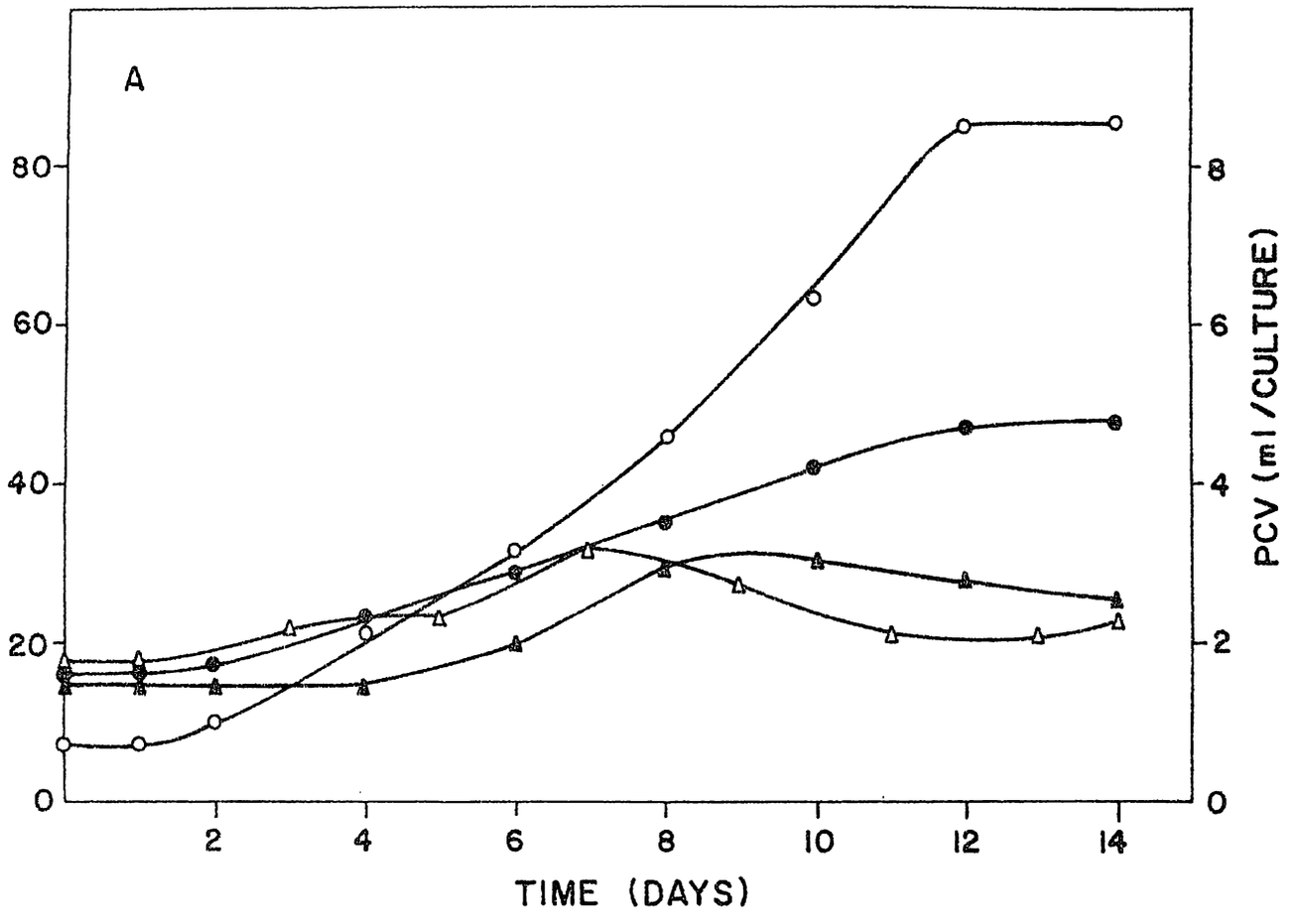
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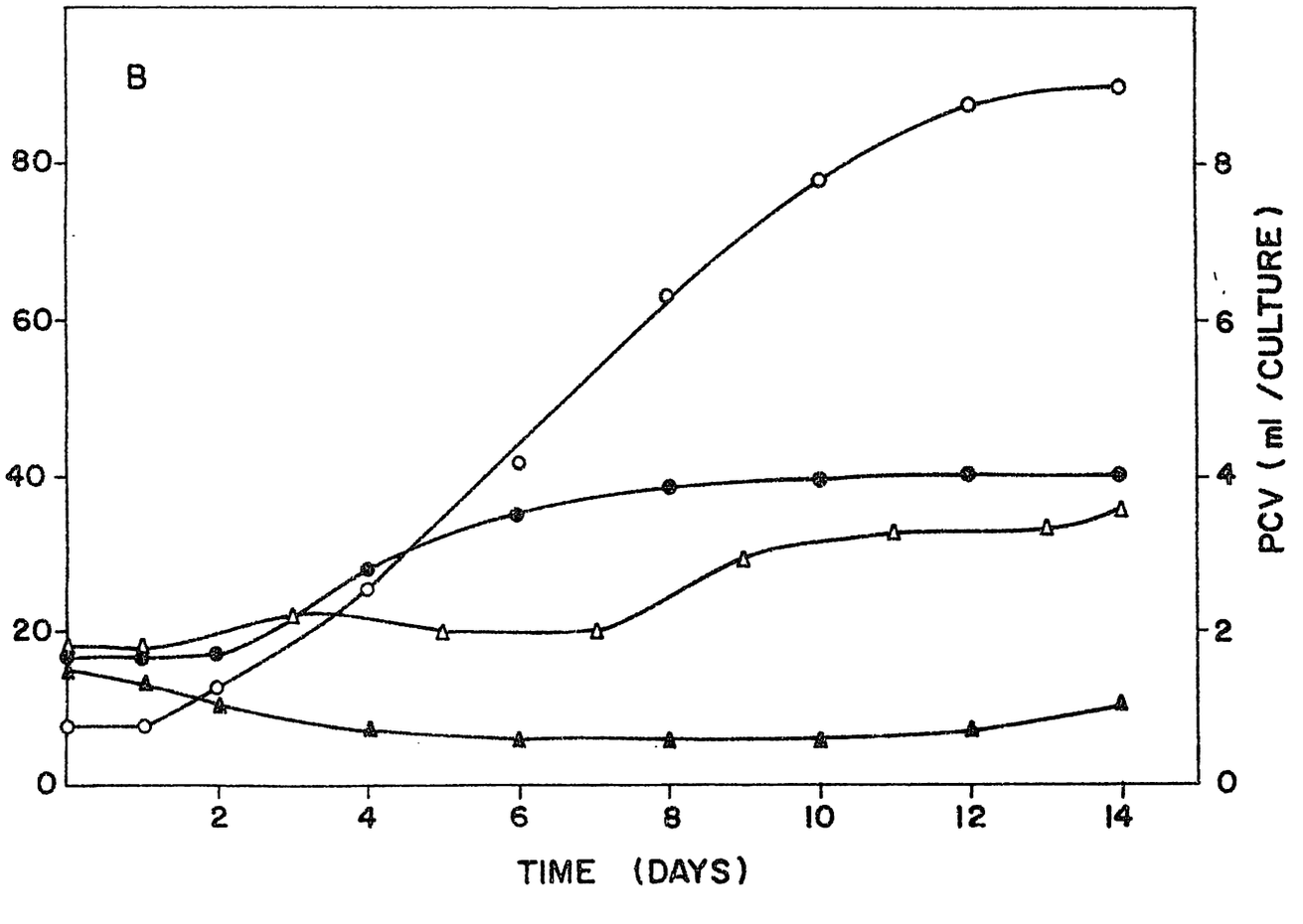
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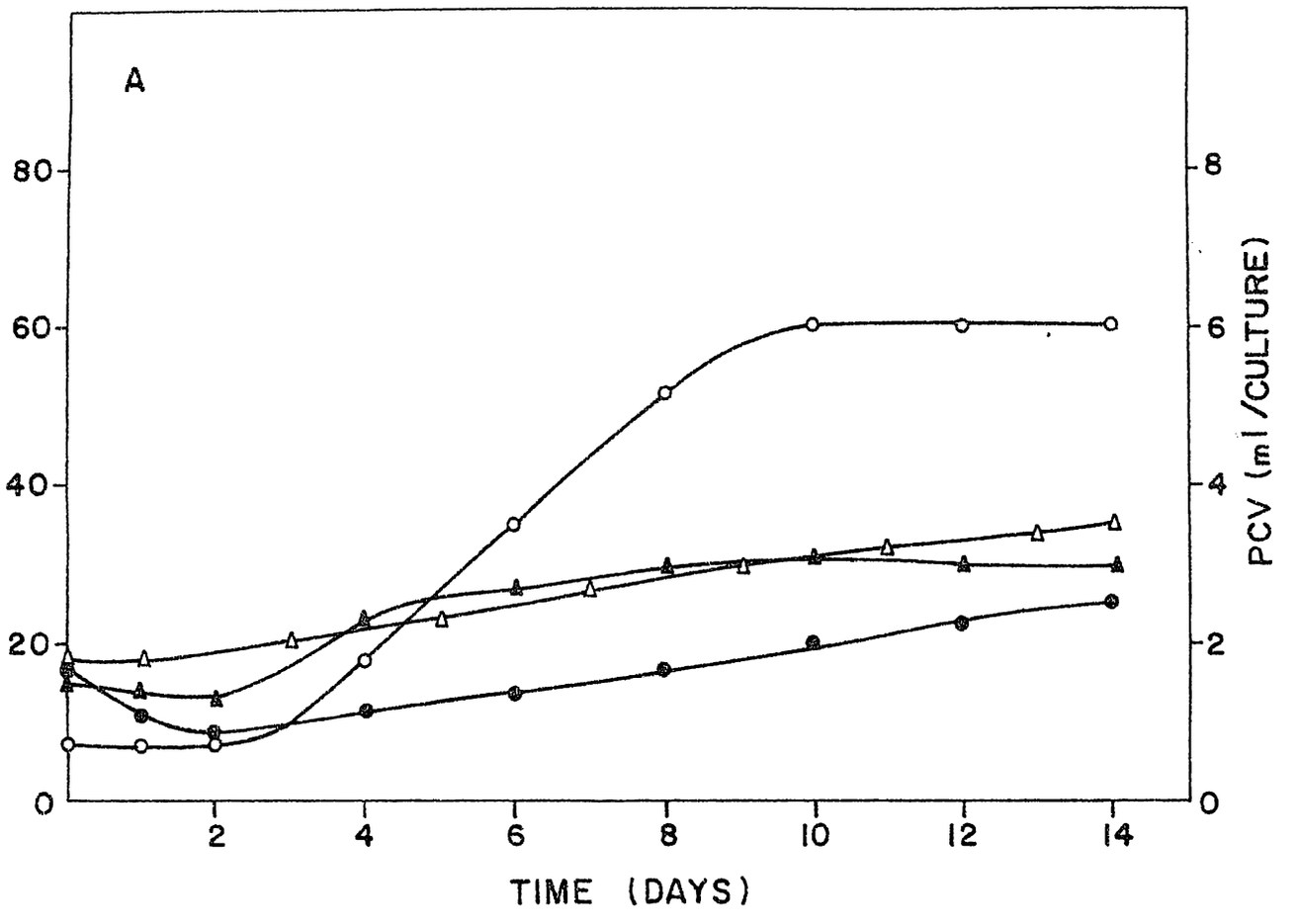
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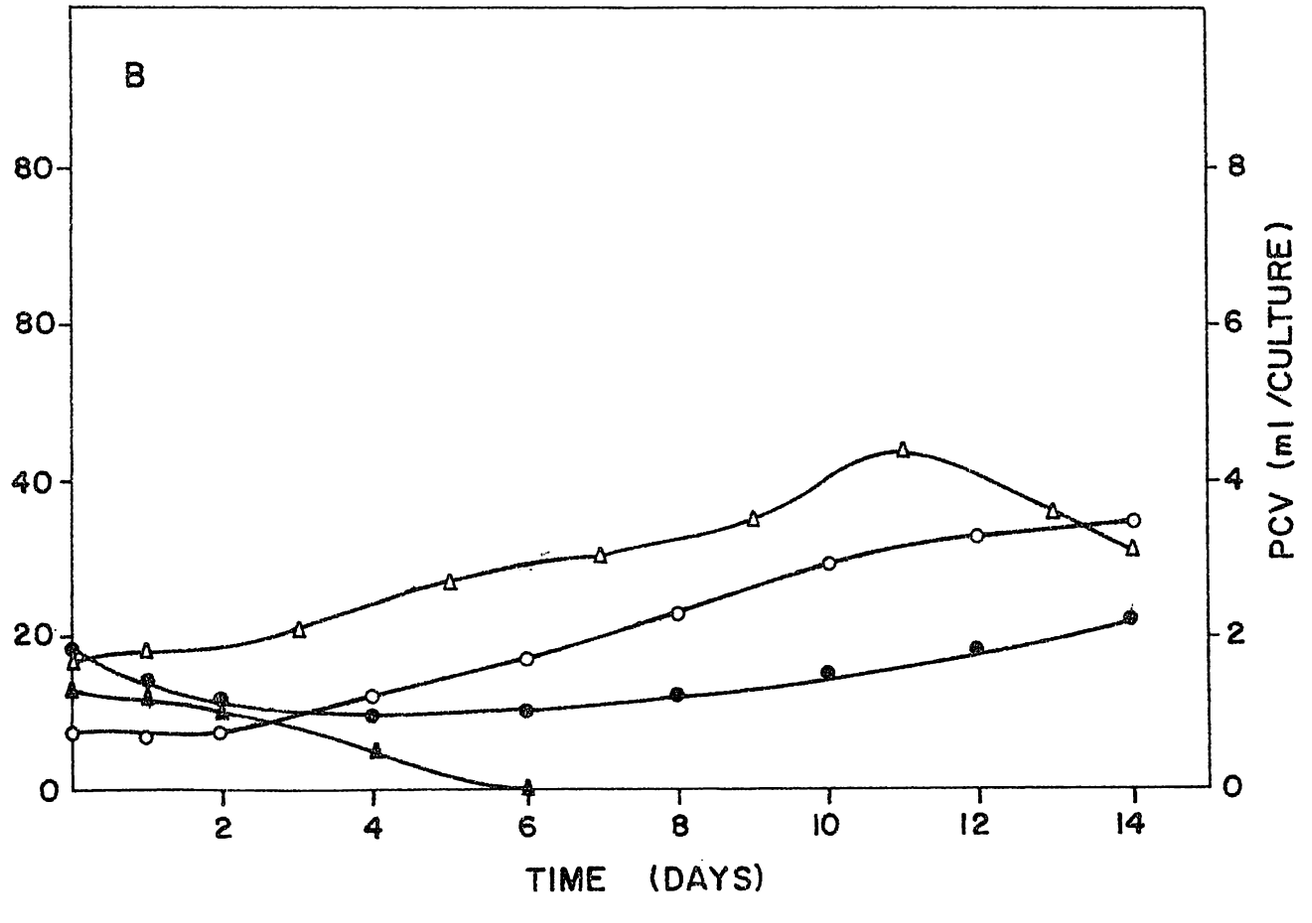
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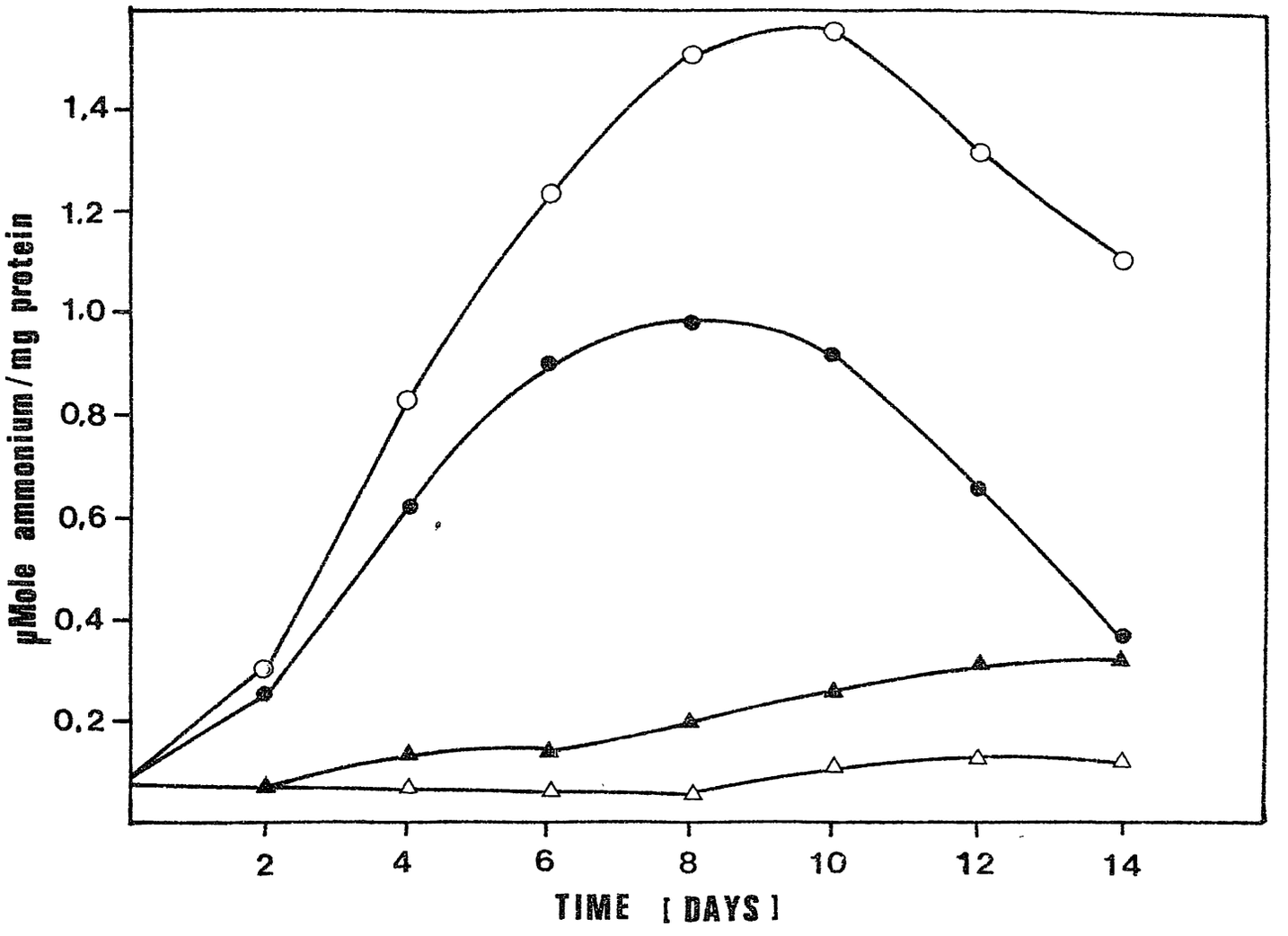
TIME (DAYS)
PCV (ml/CULTURE)


µMole ammonium / mg protein
1,4
1,2
1,0
0,8
0,6
0,4
0,2
2
4
6
8
10
12
14
TIME [DAYS]

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Glutamate synthase in greening callus of *Bouvardia ternifolia* Schlecht

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Abstract

The distribution of the two glutamate-synthase (GOGAT) activities known to exist in higher plants (NADH dependent EC 2.6.1.53, and ferredoxin dependent EC 1.4.7.1.) was studied in non-photosynthetic and green callus tissue in culture as well as in young leaves of B. ternifolia.

NADH-glutamate synthase was present in all three tissues. Using a sucrose gradient it was found in both soluble and plastid fractions of non-photosynthetic and green callus, but was confined exclusively to the chloroplast fraction in the leaves. Ferredoxin-GOGAT was found only in green tissues and was confined to the chloroplast.

Ferredoxin-glutamate synthase activity increases in parallel with the chlorophyll content of the callus during the greening process in the MS medium (nitrate and ammonium as the nitrogen sources), while NADH-GOGAT is unaffected by the greening process in this medium. Furthermore, both activities are differentialy affected by either nitrate or ammonium as the sole nitrogen source in the medium during this process. It is suggested that both GOGAT activities play different roles in the intermediate metabolism of B. ternifolia.

Key words: Bouvardia ternifolia, ferredoxin, glutamate synthase, greening callus tissue.

Introduction

Chloroplasts are important in nitrogen metabolism (Miflin and Lea 1980) since the two distinct glutamate synthase activities (NADH and ferredoxin dependent) which have been found in plants seem to be localized in these organelles (Wallsgrove et al 1979 , Matoh and Takahashi 1981) the physiological meaning of these activities is however not clearly understood. Ferredoxin-GOGAT has been related to ammonium reassimilation from the photorespiratory cycle (Somerville and Ogren 1980, Woo et al 1982); however the enzyme has also been found in white tissue (Matoh and Takahashi 1982, Susuki et al 1982, Wallsgrove et al 1982) leaving its role still open.

Callus culture is a good material for the study of certain aspects of the plant metabolism. A greening callus represents biochemical and cellular differentiation whithout tissue and organ differentiation; therefore metabolic changes related to cellular differentiation can be examined in this system (Chaleff 1981) with the advantage of greater cell homogeneity and control of the cell nutrient enviroment against etiolated leaves. Furthermore there are few reports of biochemical parameters from greening callus and leaf tissue to allow for reliable comparison. On the other hand, greening tissue culture has been recognized as a possible producer of secundary metabolites when white tissue culture is unable to do so (Dougall 1979), prompting the study of its metabolic functioning.

In this work, NADH and ferredoxin-GOGAT have been used

as markers to allow plastid differentiation in tissue culture, looking for a better understanding of the callus clulture as an in vivo system for cell differentiation studies.

Materials and methods

Tissue culture. Bouvardia ternifolia leaf callus was grown on MS medium (Murashige and Skoog, 1982) supplemented with 1 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.005 mg/l of kinetin (N^6-furfurylaminopurine) as previously descibed (Fernandez and Sanchez de Jimenez 1982). In order to induce greening, the tissue was placed on MS medium with different set of hormones: 5 mg/ml of benzyladenine (N^6-benzylaminopurine) and 0.1 mg/l of α-naphthalene-acetic acid; sucrose was reduced from 3% to 1.5%. The calli were grown under continuous white light (Slimline 39, Solar lamp, Mexico; 20 W m^{-2}, measured with a LI-185A Photometer, Lambda Instruments Co),at 28°C. The CO_2 enriched atmosphere was generated in a closed chamber using $NaHCO_3$ and citric acid as a source of CO_2 to obtain 0.1% concentration, calculated at atmospheric pressure and 28°C.

Enzyme activities. Calli were homogeneized with a 0.05 M potassium phosphate buffer (using KH_2PO_4 and K_2HPO_4) pH 7.5, 5 mM ethylendiaminoacetate (EDTA) 1 μM phenylmethylsulfonyl fluoride (PMFS), 0.1% β-mercaptoethanol (in the case of leaves and green callus 10% polyvinyl pirrolidone(PVP) w/v was added) in a Polytron homogeneizer (Kinematica GMBH Luzern) at 20 000 rpm for 20 s. The homogenate was filtered

through eight layers of cheescloth and centrifuged at 20 000 g for 15 min, the supernatant was used as the enzyme source. NADH-glutamate synthase was measured as reported in Fernandez and Sanchez de Jimenez (1982); ferredoxin-GOGAT was measured according to Arime (1979) using ferredoxin (purified according to Buchanan and Arnon 1981) as electron donor. Assay conditions were checked as to be in zero order kinetics. One unit of GOGAT activity is defined as 1 nm glutamate transformed per minute under the assay conditions. Protein content was measured by the biuret method (Gornal et al 1949), and chlorophyll content according to Arnon (1949).

Protoplasts preparation. Protoplasts were obtained either from non-photosynthetic callus induced from leaves, or from young leaves according to the method of Power and Cocking (1970) using cellulase (Onozuka R-10) and pectinase (Macerozyme R-10)(Yakult Biochemical Co, LTD. Nishinomiya Japan) as described previously (Fernandez and Sanchez de Jimenez 1982).

Cell fractionation. Freshly prepared protoplast (10^4 cells/ml) were suspended in 10 mM KCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.1 M tricine pH 7.5 at 4^oC, passed several times through a N^o 22 needle and placed on a continuous-discontinuous sucrose gradient in 0.1 M tricine buffer pH 7.5, 1 μM PMFS. The gradient consisted of 4 ml of 60% sucrose, 6 ml of a 60% to 42% sucrose linear gradient, 5 ml of 42% sucrose, 8 ml of a 42% to 25% sucrose linear gradient and 3 ml of 25% sucrose. The gradients were centrifuged in a SW-27 Spinco rotor (Beckman Instruments Inc.) at 25 000

rpm for 5 min, followed by 18 000 rpm for 30 min. Fractions of 1.5 ml were collected and dialized against 0.01 M potassium phosphate buffer, pH 7.5, 5 mM EDTA, 0.1% β-mercaptoethanol and 1 μM PMFS. Triton X-100 was added (0.1% v/v) to each fraction, and enzyme activities measured. Nitrite reductase was measured according to Losada and Paneque (1971) cytochrome oxidase according to Wharton and Tzagaloff (1967), alcohol dehydrogenase according to Kaplan (1962) and catalase according to Arnon (1949).

Differential centrifugation. Freshly prepared protoplasts broken as above, were centrifuged either at 5 000 g (callus) or 2 500 g (leaves) for 10 min. The supernatant was centrifuged at 20 000 g for 10 min in a J2-21 centrifuge (Beckman Instruments Inc.) at 4°C. The 5 000 g, 2 500 g and 20 000 g pellet fractions were resuspended in potassium phosphate buffer pH 7.5, 5 mM EDTA, 0.1% β-mercaptoethanol, 1 μM PMFS and 0.1% triton X-100. The supernatant was supplemented with EDTA and β-mercaptoethanol to have a final concentration of 5 mM and 0.1% respectively; and enzyme activities were measured in each fraction.

Results

The change of chlorophyll content, NADH-GOGAT and ferredoxin-GOGAT activities were followed in callus during the greening period in CO_2 enriched atmosphere (0.1%).

Chlorophyll content and the activities of NADH-and ferredoxin-GOGAT were measured in green callus and young green leaves. The specific activity of NADH-GOGAT was similar in

leaf tissue, non-photosynthetic and green callus tissues. In contrast ferredoxin-GOGAT activity was much higher in leaf tissue than in green callus, and it was not detectable in non-photosynthetic callus (Table 1). When ferredoxin-GOGAT activity was expressed as units per mg chlorophyll (10.5 mg chlorophyll/g fresh tissue in green leaves, 1.5 mg chlorophyll/g fresh weight in green callus), there was not correlation between activity and chlorophyll content (Table 1).

In the standard MS medium (nitrate and ammonium as nitrogen sources), chlorophyll was detectable after 6 d of incubation and its level continued to rise untill the end of the incubation period (20th day). Ferredoxin-GOGAT was not detectable untill the 8th day, and the activity reached a plateau at the end of the incubation period (20th day). NADH-GOGAT was present throughout the entire incubation period, with a slight decrease during the first 4 d (Fig. 1A).

When ammonium was used as the sole nitrogen source, there was no significant change of NADH-GOGAT activity; chlorophyll appeared later and at a much lower level compared with the standard medium. Ferredoxin-GOGAT was not detected at all (Fig. 1B). When nitrate was the nitrogen source, NADH-GOGAT tended to go down throughout the greening period. Chlorophyll appeared earlier and tended to be higher at the end of the incubation period than in the MS medium. Ferredoxin-GOGAT however, increased as in the standard MS medium (Fig. 1C).

Protoplasts were fractionated by differential centrifugation and each fraction (2 500/5 000 g, 20 000 g and supernatant fractions) were analysed for enzyme activities.

Alcohol deydrogenase was exclusively found in the supernatant fraction of the non-photosynthetic and green callus; green leaves did not have any detectable alcohol dehydrogenase activity. Nitrate reductase was found mostly in the 5 000 g pellet from non-photosynthetic and green callus and in the 2 500 g pellet of the leaves (Table 2).Ferredoxin-GOGAT was exlusively confined in the 2 500/5 000 g fractions in the green tissues. On the other hand, NADH-GOGAT was distributed both in the 5 000 g fraction and in the supernatant of the non-photosynthetic and green callus, while it was found exclusively in the 2 500 g fraction of the green leaves (Table 2).

These results were confirmed by continuous-discontinuous sucrose gradient experiments (Fig. 2 A-B). The following markers were used: cytochrome oxidase for mitochondria, nitrite reductase for plastids, catalase for peroxisomes, alcohol dehydrogenase for cytoplasm and chlorophyll for chloroplasts as have been used previously (Quail 1979). Ferredoxin-GOGAT activity could not be detected in green callus along the gradient fractions probably because of the low levels of this enzyme in this tissue. Protoplasts from young leaves were used instead. In this tissue, both GOGAT activities were found exclusively within the peak containing chlorophyll (Fig. 2B). In the gradient from non-photosynthetic callus, NADH-GOGAT was found both as a broad peak together with nitrate reductase activity and in the upper part of the gradient together with the alcohol dehydrogenase activity (Fig. 2A). In the green callus the ditribution of this

was the same as in the non-photosynthetic callus (data not shown). In all cases, recuperation from the gradient was between 90 and 100 %, except for cytochrome oxidase whose recuperation from the gradient was around 80%.

A differential effect was observed when protoplasts from non-photosynthetic and green callus were incubated with cycloheximide under conditions of 90% protein synthesis inhibition (Table 3). The soluble NADH-GOGAT was strongly diminished in both types of calli (37.4% in non-photosynthetic callus and non detectable activity in green callus); while the plastidic enzyme was only mildy affected (75% in non-photosynthetic callus and 67.6% in green callus).

Discussion

The effect of light on the two GOGAT activities during the greening of B. ternifolia callus is similar to that reported previously by Matoh and Takahashi (1982) in greening pea shoots and Wallsgrove et al (1982) in greening barley and pea leaves. However the NADH-GOGAT activity is higher than the ferredoxin-GOGAT activity in greening calli under all conditions used, while the opposite is observed in B. ternifolia leaf tissue and those mentioned above (Table 1).

The nitrogen source influences the two GOGAT activities in a different way. If ammonium is the sole nitrogen source, ferredoxin-GOGAT does not appear at all; if nitrate is the only nitrogen source, this enzyme behaves much as in the standard medium. These results suggest a dependence of ferredoxin-GOGAT activity upon chloroplast differentiation

although there does not seem to be a strict correlation between ferredoxin-GOGAT activity and chlorophyll content (Table 1). NADH-GOGAT on the other hand, seems to be independent of the appearance of chloroplasts. However nitrate in the medium affects the activity of NADH-GOGAT. This could be explained if exists competition for reducing power between 2-oxoglutarate and nitrate, and if the availability of reducing power affects NADH-GOGAT expression. This is consistent with the data of Matoh and Takahashi (1982) who have shown that NADH-GOGAT is more abundant during the early leaf growth, while ferredoxin-GOGAT becomes predominant during leaf maturation when photosynthesis activity has been fully developed.According to this, green B. ternifolia callus wuold be similar to young leaf as is also indicated by the low chlorophyll content of the two tissues and its dependence on external sucrose source.

Both ferredoxin- and NADH-GOGAT activities have been found in root and leaf plastids (Matoh and Takahashi 1981 ; Wallsgrove et al 1979) and we have also found the activities in chloroplasts of B. ternifolia leaves (Fig 3). However, in our hands the differential centrifugation and the continuous-discontinuous sucrose gradient showed that in the non-photosynthetic callus NADH-GOGAT is also present (about 40% activity) in the cytosol. This fraction does not seem to come from broken plastids since only around 4% of nitrate reductase activity was found contaminating the supernatant fraction in the differential centrifugation experiments (Table 2), and no nitrite reductase was found in the super-

natant fractions from the sucrose gradient (Fig 2B). Furthermore both activities are affected differentialy by cycloheximide: the soluble enzyme is strongly inhibited (Table 3), whereas the plastidic enzyme seems to be less affected. This suggests that both enzymes are different entities or that both are differentially regulated at the level of protein synthesis. Further work is required to find out whether these two activities actually represent different isoenzymes.

Table 1. Ferredoxin- and NADH-GOGAT activities in young leaves, non-photosynthetic and green callus after 15 days of subculture.

	GOGAT activity (nMole glutamate min^{-1})		
	Ferredoxin -GOGAT		NADH-GOGAT
	per mg protein	per mg chlorophyll	per mg protein
Non-photosynthetic callus	0.0	0.0	5.4 (0.61)[a]
Green callus	0.8 (0.2)	3.33 (0.62)	4.4 (0.53)
Leaves	4.5 (0.33)	3.2 (0.22)	4.2 (0.21)

[a] Each value represents the mean $\pm$ SD, n= 4

Table 2. Differential centrifugation of broken protoplasts from cell cultures and young green leaves.

	[a]Alcohol dehydrogenase	[b]Nitrite reductase	[c]NADH-GOGAT	[c]Ferredoxin-GOGAT
Non-photosynthetic callus				
5 000 g pellet	ND[d]	4.25 (0.36)[e]	0.21 (0.06)	-
20 000 g pellet	ND	0.66 (0.14)	ND	-
Supernatant	5.8 (0.4)	0.21 (0.02)	0.28 (0.06)	-
TOTAL	6.2 (0.3)	4.81 (0.62)	0.51 (0.12)	-
Green callus				
5 000 g pellet	ND	4.02 (0.44)	0.28 (0.02)	0.42 (0.04)
20 000 g pellet	ND	0.15 (0.02)	ND	ND
Supernatant	3.1 (0.36)	0.22 (0.08)	0.28 (0.03)	ND
TOTAL	3.3 (0.42)	4.5 (0.4)	0.55 (0.04)	0.42 (0.04)
Leaves				
2 500 g pellet	ND	5.2 (0.6)	0.35 (0.12)	0.92 (0.12)
20 000 g pellet	ND	0.82 (0.12)	ND	ND
Supernatant	ND	ND	ND	ND
TOTAL	ND	6.1 (0.42)	0.31 (0.08)	1.02 (0.02)

[a] in μmol NADH $min^{-1}/10^3$ protoplasts

[b] in μmol NO_2^- $min^{-1}/10^3$ protoplasts

[c] in μmol glutamate $min^{-1}/10^3$ protoplasts

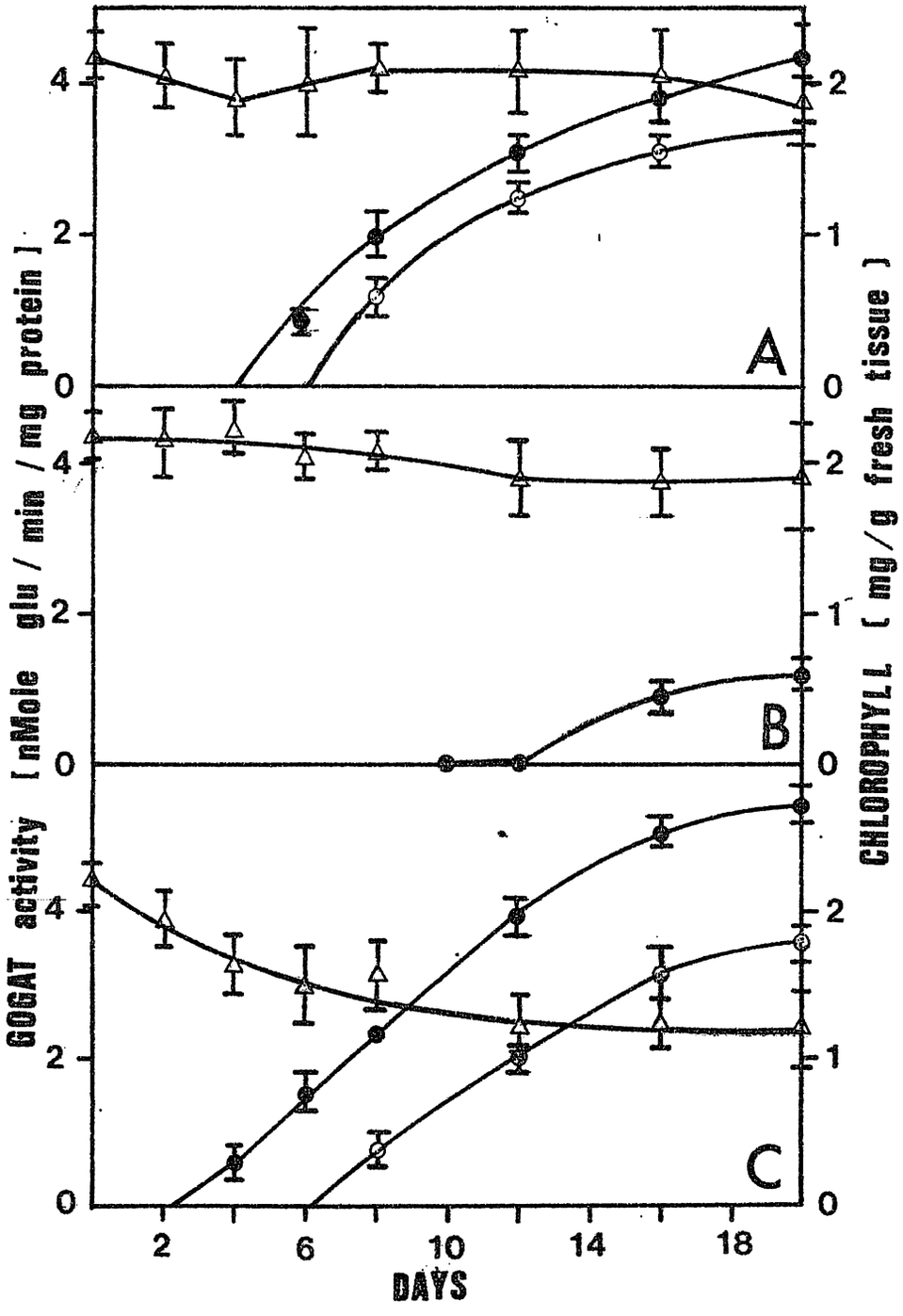
[d] ND= not detectable

[e] Each value represents the mean $\pm$SD n= 4

Table 3. Inhibition of NADH-GOGAT activity in non-photosynthetic and green callus. Freshly prepared protoplasts (10^4 cells/ml) were incubated with cycloheximide (10 μg/ml) for one hour; intact protoplasts were recovered and then differentialy centrifuged.

Protoplast source	Fraction	Units of GOGAT mg protein^{-1}		
		Control	Cycloheximide	% recovery
Non-photosyntetic callus	5 000 g pellet	8.41 (0.3)	5.9 (0.52)	75 %
	Supernatant	5.64 (0.2)	2.11 (0.24)	34.4
Green callus	5 000 g pellet	9.16 (0.92)	6.2 (0.43)	67.6
	Supernatant	3.6 (0.4)	0.0	0.0

[a]Each value represents the mean $\pm$ SD n= 4


GOGAT activity [nMole glu / min / mg protein]
CHLOROPHYLL [mg / g fresh tissue]
A
B
C
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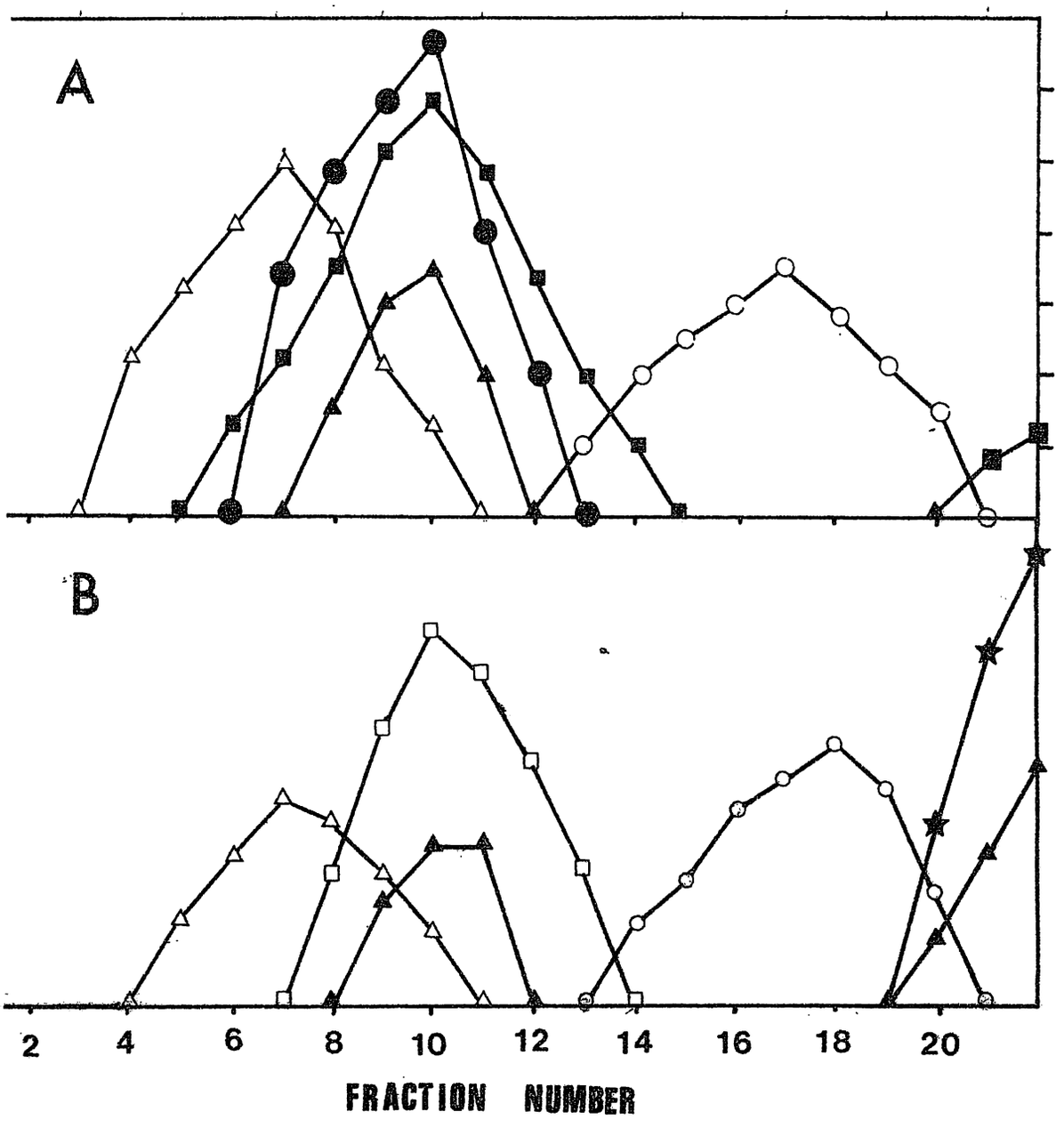
2
4
1
DAYS
2
6
10
14
18


A
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16
18
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FRACTION NUMBER

Legend to figures

Fig. 1 A-C. Changes in chlorophyll content (●), NADH-GOGAT (Δ) and ferredoxin-GOGAT (○) activities during the greening of callus in standard MS medium (KNO_3 and NH_4NO_3 as nitrogen sources) (A); medium with 10 mM NH_4Cl, 10 mM sodium citrate (pH 5.8) (B) and medium with 20 mM potassium nitrate (C) as the only nitrogen source. Each point represents the mean value of al least 4 replicate experiments. Vertical bars stand for SD.

Fig. 2 A-B. Organelle separation in a sucrose continuous-discontinuous gradient. Protoplasts were prepared from young green leaves (A) or white callus (B). The following enzyme activities were assayed: NADH-GOGAT (nMole glu min^{-1}) (▲), ferredoxin-GOGAT (nMole glu min^{-1}), (●), catalase (ΔA_{240} min^{-1} x 10) (Δ), cytochrome oxidase (mg cytochrome min^{-1} x 10) (○), alcohol dehydrogenase (nMole NADH min^{-1} x 10) (★), nitrite reductase (nMole NO_2^- min^{-1} x 10) (□), and chlorophyll content (g x 10) (■).

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REGULATION OF FERREDOXIN-GOGAT IN ISOLATED CHLOROPLASTS

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Abstract

The mechanism regulating ferredoxin-GOGAT activity in B. ternifolia chloroplasts was investigated. Chloroplasts were isolated under conditions where ferredoxin-GOGAT activity was unaltered during 3 h of incubation in continuous light. In darkness, this activity decreases to about half the original level. Extraction with sulphydryl reducing chemicals such as ditiotreithol and mercaptoethanol did not revert this phenomenon; neither preincubation with reducing sustrates such as ferredoxin, NADH or NADPH. These chloroplasts exported glutamate in light during the incubation period, when glutamine, ammonium, and 2-oxo-glutarate were added to the system. The activity of ferredoxin-GOGAT under light or in darkness was the same with these sustrates. Addition of DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) to the system showed that the expression of ferredoxin-GOGAT activity has a dependence on the electron transport.

The incorporation of ^{14}C aminoacids into proteins was effectively inhibited by chloramphenicol in the isolated chloroplasts. Under this conditions we detected a rapid decay of ferredoxin-GOGAT activity, this followed first order kinetics with a half life of 1 h 43 min. Inactive enzyme was detected by antibody precipitation when isolated chloroplasts were incubated under conditions of activity decay (either in darkness or protein synthesis inhibition) showing a partial inactivation of the enzyme rather than a complete degradation.

Introduction

Two activities of glutamate synthase, one NADH dependent (EC 2.6.1.53) and the other ferredoxin dependent (EC 1.4. 7.1.) have been reported for most plants (Miflin 1980). It has been shown that both are different protein entities (Susuki et al 1984). Ferredoxin-GOGAT has been found exclusively within the chloroplasts of green tissues (Wallsgrove et al 1979, Matoh and Takahashi, 1981, Murillo and Sanchez de Jimenez 1983) and its function has been related to the assimilation of ammonium liberated during the conversion of glycine to serine in the photorespiratory cycle; ferredoxin-GOGAT supplies glutamate for both glutamine synthetase and for transamination of glyoxilic acid to form glycine (Keys et al 1978). This conclusion is mainly based on the dependence of photorespiration on the presence of glutamine synthetase activity (Franz et al 1982, Larsson et al 1982, Nishimura et al 1982), furthermore, ferredoxin-GOGAT mutants of Arabidopsis are unable to survive under photorespiratory conditions (Somerville and Ogren 1980).

Despite all these data, the function of ferredoxin-GOGAT is still not fully understood, since this enzyme has also been reported to be present in non-photosynthetic tissues (Matoh and Takahashi 1982, Wallsgrove et al 1982) and is very active in C-4 plants (Matoh et al 1979) where photorespiration is undetectable or very low (Chollet and Ogren 1975). On the other hand, glutamate as the transaminating aminoacid in the glyoxilic-glycine reaction has been

chalanged (Betsche 1983).

Therefore it was thought to be important to study the regulation of ferredoxin-GOGAT activity in isolated B. ternifolia chloroplasts in order to fully understand the physiological role of this enzyme.

Previous work in our laboratory have shown that ferredoxin-GOGAT activity is not present in B ternifolia non-photosynthetic callus and it appears as the tissue becomes green, moreover it is exclusively confined in chloroplasts of both green tissue and leaves (Murillo and Sanchez de Jimenez 1983).

Materials and methods

Plant material. B.ternifolia leaves were obtained from the University Botanical Gardens. Leaves were washed with tap water, cut into small pieces and then places in a neutralized solution of 0.1 mg/l of kinetin (N^6-furfuryl-aminopurine), untill they were used.

Chloroplasts isolation. Chloroplasts were isolated from young leaves. All steps were done at 4^oC. The pieces of leaves were homogeneized in a buffer solution containing 0.3 M sorbitol, 20 mM tricine, pH 8.4, 10 mM ethilendiaminetetraacrtate (EDTA), 10 mM $KHCO_3$ and 0.1% bovine serum albumin, in a Polytron homogeneizer (Kinematica BMGH Luzern) for 15 seconds at 20 000 rpm. The homogenate was centriguged for two minutes at 1 000 rpm on a MSE centrifuge and then the supernatant was inmediately centrifuged for 15 min at 3 000 rpm in the same centrifuge.

The chloroplasts were washed once in the same solution. The sediment is crude chloroplasts; these were resuspended in the homogeneization solution to have a chlorophyll concentration of 300 to 400 µg chlorophyll/ml, and then inmediately used.

Assay for ferredoxin-GOGAT activity. The assay was practically the one used by Susuki et al (1982). At different times two ml of chloroplasts were recuperated by centrifugation (3 500 for 3 min in MSE centrifuge) and then resuspended in a buffer containing 0.05 M potassium phosphate pH 7.5, 0.1% β-mercaptoethanol, 10 mM EDTA and 1 µM phenylmethylsulphonyl fluoride (PMFS) and 0.1% Triton X-100. The broken chloroplasts were centrifuged for 20 min at 3 500 rpm in the MSE centrifuge. The supernatant was used as the source of the enzyme. Chlorophyll was measured prior to chloroplast breakage according to Arnon (1949) and proteins were measured from the postbreakage supernatant by the buret method (Gornall 1949). The assay mixture was composed of 5 µmol 2-oxoglutarate, 5 µmol glutamine, 0.02 µmol ferredoxin (purified form spinach leaves according to Buchanan and Arnon 1968 wuth a A_{420}/A_{276} value between 0.28-0.34), 9 mol $Na_2S_2O_4$ (in 50 µl dissolved in 190 mM $NaHCO_3$) and 100-200 µl of the crude enzyme. The reaction was begun with the sodium dithionite and it was incubated for 20 minat 34°C in a water shaker (120 rpm). This conditions were experimantally confirmed to correspond to zero order kinetics. The reaction was stopped boiling the assay mixture for 2 min. The mixture was

then cooled and passed through an anionic exchange resin (2x1,5 cm) (AG 1x8 Dowex). It was then washed with 5 ml of water and then the glutamate was eluted with 2 ml of 1 N acetic acid. The eluate was adjusted to pH 7 with KOH. Glutamate was estimated by the ninhydrin method (Moore and Stein 1948).

Glutamate determination. One ml of incubated chloroplasts were centroguged at 4°C at 5 000 rpm in a J2-21 centrifuge (Beckman Instruments Inc) for 15 min. The supernatant was passed through the anionic interchange resin as described above.

Protein synthesis inhibition. The isolated chloroplasts were suspended and 10 µC of ^{14}C aminoacid mixture was added (100 µl) in 5 ml of chloroplasts suspension (chlorophyll concentration 350 µg/ml). The assay was incubated at different times in white light. The chloroplasts were recuperated by centrifugation, washed and then inmediately broken (see above), the total ^{14}C-aminoacid uptake was measured from this preparation, ^{14}C-aminoacid incorporation into proteins was measured from the 5% TCA precipitate, this was washed twice with cold ethanol and then counted in a Perkin-Elmer scintilation counter.

Enzyme purification. Chloroplasts obtained as described above from 100 g of B. ternifolia leaves were broken in a small volume of 50 mM potassium phosphate buffer pH 7.2 10 mM β-mercaptoethanol, 5 mM EDTA. 1 µM PMFS and 0.1% Triton X-100 by vortexing them during 2 min; they were centrifuged at 18 000 rpm (J2-21) for 30 min. Acetone was

added (-15°C) to have a 35% solution and it was kept at -15°C for two hours. The precipitate was resuspended in a small volume of 5 mM EDTA, 5 mM β-mercaptoethanol, 1 mM EDTA, 1 μM PMFS and then dialized overnight against the same buffer. The dialized enzyme was loaded over a 20x1 cm column with DEAE-cellulose previously washed with the same buffer. The loaded column was washed with 50 ml of the same buffer, and then with a 40 to 60 mM NaCl gradient (40 ml), 5 ml fractions were taken, and those with the enzyme activity (50.1 to 55 mM) were put together, dialized against water overnight, liofilized and then resuspended in a small volume of isotonic solution.

Antibody preparation. Ferredoxin-GOGAT (375 μg) were emulsified with the complete Freund's adyuvant. This was administred subcutaneously to a rabbit. The booster injection was performed 4 weeks later by the same amount of the antigen through the ear vein. One week after the second administration, the serum was collected by heart punction and the globin fraction was purified by 0% to 35% saturated ammonium sulfate fractionation. The IgG was dissolved in 150 mM borate buffer pH 8.1 containing 0.8% NaCl and then dialized against the same buffer. Immunotitration was performed by incubating different amounts of antibody with 5 units of enzyme activity for 30 min at room temperature and then for 12 h at 4°C. The antigen-antibody complex was removed by centrifugation at 10 000 rpm in a L-50 centrifuge (Beckman Instruments Inc,) for 20 min. Glutamate synthase was assayed in the supernatant fraction.

Results

Isolated chloroplasts from young leaves of B. ternifolia are functional at least for 3 h when they are isolated as described in materials and methods. They showed a low mitochondrial contamination as measured by cytochrome oxidase activity. The integrity of the chloroplasts preparation assayed by the ferricyanide-dependent O_2 evolution method (Lillye et al 1975) was around 80%. Ferredoxin-GOGAT activity was kept almost constant through the 3 h light incubation period, with a slight but consistent increase in activity. In darkness however, this activity fell to half its initial value after the same incubation period. Re-illumination of the chloroplasts resulted in some recuperation of ferredoxin-GOGAT activity (Fig 1).

This phenomenon could be explained if the enzyme activity were dependent on the internal pH or Mg^{++} concentration changes know to ocurr in the dark-light cycle. To test this possibility, chloroplasts samples were broken at different pH or Mg^{++} concentrations, incubated for 15 min and then the change in enzyme activity were recorded (Figure 2). The changes found were not of the amount expected to account for this phenomenon.

Dark incubated chloroplasts were broken with sulphydryl reducing chemicals to search for possible inactivation due to dark oxidation of sulphydryl groups; neither mercaptoethanol nor dithiotreithol could reverse the dark inactivation (table 1). The presence of ferredoxin, NADH or NADPH were also unable to produce enzyme reactivation

(table 1)

The effect of different metabolites of the glutamate production within the chloroplasts was tested in light and darkness. Chloroplasts were incubated for 3 h in light with either the GOGAT sustrates (glutamine and 2-0x0glutarate) (Fig 3A) the primary GS/GOGAT cycle sustrates (ammonium and 2-oxoglutarate) (Fig 3B) or the three metabolites (ammonium, glutamine and 2-oxoglutarate) (Fig 3C), in all cases 1 mM malate was added to assure optimum amounts of glutamate production. The glutamate exported to the medium was recorded during the incubation period. As expected, the isolated chloroplasts excreted glutamate to the medium in the presence of the sustrates, while the GOGAT activity remained constant throughout. The experiment with all sustrates (ammonium, 2-oxoglutarate and glutamine) was carried out as above but in darkness. Under these conditions, only a slight amount of glutamate was produced and ferredoxin-GOGAT dramatically declined as previously observed.

Since ferredoxin-GOGAT activity seems to be light dependent, the effect of a specific inhibitor of photosynthesis was analysed. Chloroplasts were pre-incubated with DCMU (3-(3,4-dychlorophenyl)-1,1-dimethylurea) for 3 min in 0.17 mM ethanol, (this treatment completely stopped O_2 evolution in light) and incubated in light or darkness for 3 h as above. Ferredoxin-GOGAT activity was followed during this period (Fig 4). In light only 20 % of the activity was lost at 3 h, while in darkness the activity was lost

the 50% that had been observed in the untreated chloroplasts (Fig. 4). The addition of the three sustrates to the medium (ammonium, 2-oxoglutarate and glutamine) in the DCMU light system, causes small glutamate production in amounts similar to those obtained in darkness, suggesting a photosynthetic dependence for the in vivo activity of the enzyme.

The lost of enzyme activity in the darkness could be a consecuence of the protein synthesis decrement produced under this conditions. Therefore, the effecto of protein synthesis over ferredoxin-GOGAT activity was analysed.

The ability of the chloroplasts to take free aminoacds and incorporate them into proteins was tested. A mixture of ^{14}C-aminoacids were added to the incubation medium. At different times, ^{14}C-aminoacid pools, as well as ^{14}C-aminoacid incorporation into TCA precipitable material were measured. The profiles of figure 5 show that a plateau around 2 h is reached for the ^{14}C-aminoacid incorporated into proteins. When chloramphenicol is added to the system at a concentration of 50 μg/ml, protein synthesis is stopped wuthin an hour (Fig 5). Thus, to test the effect of the inhibition of protein synthesis over the ferredoxin-GOGAT activity, the chloroplasts were preincubated with chloramphenicol for one hour and then they were incubated for two more hours with the ^{14}C-aminoacids.

The ferredoxin-GOGAT activity showed a decrease which followed a first order kinetics (Fig 6). A stright line with a slope of 0.184 is obtained when the logarithm of

these values are plotted against time. This corresponds to a catalitic constant of 0.4048 h^{-1} and a half life of 1.72 h (1 h 43 min). The decay on the enzyme activity could correspond to the enzyme itself or to any other protein directly related to its activity. To clarify this point, the enzyme was purified following chloroplast isolation, acetone precipitation and ion exchange chromatography through DEAE-cellulose. A 350 fold purification of the enzyme was achived (Table 2). The antibody preparation was titrated against ferredoxin-GOGAT activity from freshly prepared chloroplasts. It was found a linear relationship between the volume of antibodies and the lost of ferredoxin-GOGAT activity Chloroplasts containing five units of ferredoxin-GOGAT activity were incubated in darkness, light, and light with chloramphenicol. At different times during the incubation period, samples were taken and the broken chloroplasts were treated with antibodies that precipitate five units of activity at zero time; thus, at this time no activity was detected in either of the samples. However one hour later, chloroplasts with the inhibitor of protein synthesis, showed some activity ; at two hours, the dark incubated chloroplasts had some activity too, and at three hours even the light incubated chloroplasts had activity in the presence of the antibodies The two former continued with the increase in activity up to 3 h. This shows the presence of inactivated enzyme competing with the antibodies added.

Discussion

The chloroplasts preparation used in this work were stable, active in their metabolic functions and capable of protein synthesis for the period of time studied (Fig 6), similarily to other reported system (Fish and Jagendorff 1982). Its transport system is also undamaged as judged by the aminoacid uptake (Fig 6) and by the transformation of ammomium, 2-oxoglutarate and glutamine taken from the medium and followed by glutamate export. The excretion of this metabolite showed to be malate dependent as previously dexcribed (Dry and Wiskish 1983, Woo and Osmond 1982) (Fig 4 and 5).

It has been found previously that ferredoxin-GOGAT is exclusively contained in the chloroplasts of young B. ternifolia leaves (Murillo and Sanchez de Jimenez 1983). Therefore these functional isolated chloroplasts are sutable system to study the ferredoxin-GOGAT regulation. The dramatic decrease of GOGAT activity observed when chloroplasts are incubated in darkness (Fig 5), indicates a strong regulation of the enzyme by light/dark changes as has been shown for other chloroplastic enzymes (Buchanan 1980). Ferredoxin-GOGAT however seems not to be regulated through changes of internal pH or Mg^{++} concentration (Fig 2). These should ocurr in short times of exposure as expected by this type of regulation (Werden et al 1975, Portis et al 1977).

Another mechanism of light control is through the formation of reduced sulphydryl groups after light exposure.

Ferredoxin-GOGAT has been shown to be activated by this mechanism in Chlorella (Tischner 1981); however, the B. ternifolia enzyme is neither reactivated by mercaptoethanol nor by dithiotreithol as the Chlorella does (Slovacek and Vaughn 1982). Therefore a different regulatory system between the same enzyme in algal and higher plant is opperative. The reducing power generated directly from photosynthesis (as ferredoxin or NADPH) did not reactivate the enzyme, after incubation with the crude extract from the broken chloroplasts. However, it should be noted that the in vivo activity of the enzyme seems to be regulated by the availability of the reduced cofactor (ferredoxin) since under conditions of photosynthesis inhibition (DCMU in light) the enzyme decreases 20% of its original value and the glutamate export is highly reduced (Fig 5). Therefore, photosynthesis seems to regulate the enzyme activity either through the enzyme stability into the chloroplast or through the lack of energy required for protein synthesis; although a low non-photosynthetic generation of ATP in darkness is know to be functioning for protein synthesis (Ramirez et al 1968, Kow et al 1982). This explanation asumes a fast turnover of the enzyme and the dramatic lost of enzyme activity in the experiment of inhibition of the protein synthesis (Fig 7) seems to support this hipotesis. However, we found activity when we added antibodies against ferredoxin-GOGAT to the incubated chloroplasts with inhibition of their protein synthesis. This might have two explanations; one could be the presence of inactive enzyme,

partialy degraded, which still kept antigenic determinants recognizable by the antibody. In this case the enzyme has a very fast turnover (1 h 43 min), if it is compared with other plant enzymes (Oaks et al 1972). The other explanation needs the presence of another protein with a fast turnover, that is needed to keep the stability of the ferredoxin-GOGAT. In any case, the experiments show a regulatory mechanism dependent on light, and on de novo protein synthesis.

The fact that this enzyme is regulated by light, questions its postulated role as being the responsable for ammonium assimilation, since this metabolite is produced and taken by chloroplasts even in darkness (Woo et al 1982). So, its main function seems to be related with the flux of reducing power from the chloroplasts to the cytoplasm or to the mitochondria during the light periods.Glutamate would be the carrir of the reducing power, and this could be converted to NADH by Glutamate dehydrogenase in the cytoplasm or in the mitochondria. In darkness, the export of reducing power would be done by dihydroxiacetone phosphate derived from starch degradation (Kow et al 1982). To keep the ferredoxin-GOGAT needs, glutamine could be either taken directly from the xilem and then transported into the chloroplasts, or synthetized from cytosolic glutamine synthetase (Barber and Thurman 1978), or produced within. This mechanism could signify a metabolic link to regulate the flow of reducing power from the chloroplast to the rest of the cell with a close regulation by light.

TEST MATERIAL ADDED	ACTIVITY (nMole glu / mg chlorophyll)
Ferredoxin-GOGAT, 3 h light	4.5 (0.0)
Ferredoxin-GOGAT, 3 h darkness	2.25 (0.0)
Dithiotreithol 1 mM	2.52 (0.22)
Dithiotreithol 5 mM	1.88 (0.66)
Mercaptoethanol 1 mM	2.2 (0.2)
Mercaptoethanol 10 mM	2.7 (0.3)
Ferredoxin 20 g	2.3 (0.3)
NADH 10 mM	2.25 (0.25)
NADPH 10 mM	2.4 (0.2)

Table 1. Incubation of ferredoxin-GOGAT with reducing chemicals. Broken chloroplasts were incubated for 15 min. Chloroplasts were incubated in darkness for 3 hours. Each value in parenthesis represents the SD with n=5.

	Volume (ml)	Total activity (nMole glu/min)	Recovery (%)	Protein (mg)	Specific activity (Units/mg prot)	X purification
Crude extract	-	-	-	-	2.8	1
Broken chloroplasts	25	488	-	62.5	7.8	2.78
Acetone precipitation	5	402	82.3	14.35	28	10
DEAE-cellulose	10	254	52.2	0.26	980	350

Table 2. Enzyme purification.

Legend to figures

Fig. 1 Ferredoxin-GOGAT activity from isolated chloroplasts incubated in light (o) and darkness (●) conditions. Bars represent the SD with n=6. Dark incubated chloroplasts were illuminated after 2 h (▲).

Fig. 2 Effect of pH and Mg^{++} ions on ferredoxin-GOGAT from chloroplasts. For the pH effect, chloroplasts were broken with 0.05 M phosphate buffer at different pH, 0.1 % β-mercaptoethanol, 10 mM EDTA, 1 μM PMFS and 0.1% Triton X-100; pH was adjusted prior to the enzyme assay (o). For the Mg^{++} effect, chloroplasts were broken with the 0.05 M potassium phosphate buffer pH 7.5, 0.1% βmercaptoethanol, 3 mM EDTA, 1 μM PMFS and 0.1% Triton X-100; Magnesium ions were adjusted with $MgCl_2$.

Fig. 3 Glutamate production (●) in isolated chloroplasts, and recoverable ferredoxin-GOGAT activity (o) in broken chloroplasts with different added sustrates: gln (1mM), 2-oxoglutarate (1mM) in light (A); NH_4Cl (1mM), 2-oxoglutarate (1mM) in light (B); NH_4Cl (1mM), 2-oxoglutarate (1mM), glutamine (1mM) in light (C); and the same as (C) but in darkness (D). All media were supplemented with 1 mM malate (all reagents were neutralized at pH 7).

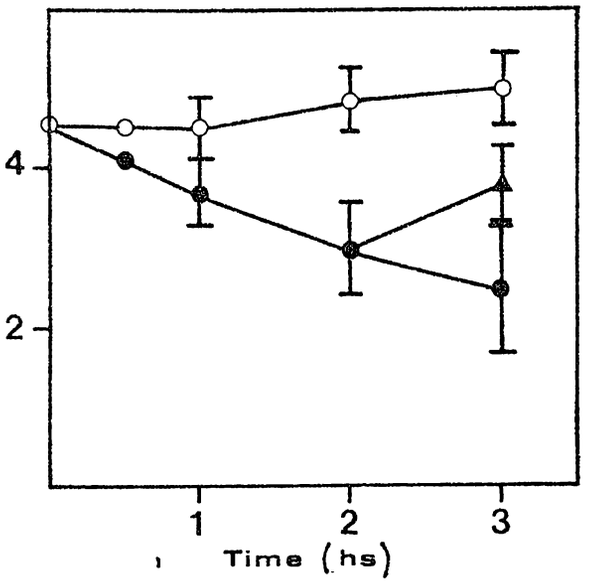
Fig. 4 Effect of DCMU on ferredoxin-GOGAT (○) and glutamate production (●) in illuminated chloroplasts, and ferredoxin-GOGAT in dark chloroplasts (▲). To measure the effect of DCMU in light over glutamate production, the medium was supplemented with NH_4Cl (1mM), glutamine (1mM), 2-oxoglutarate (1mM) and malate (1mM). Ethanol (0.17mM) was added to permit DCMU to penetrate the chloroplast. DCMU inhibited O_2 uptake completely after 3 min of preincubation. Ethanol per se has a very slight effect on the isolated chloroplasts.

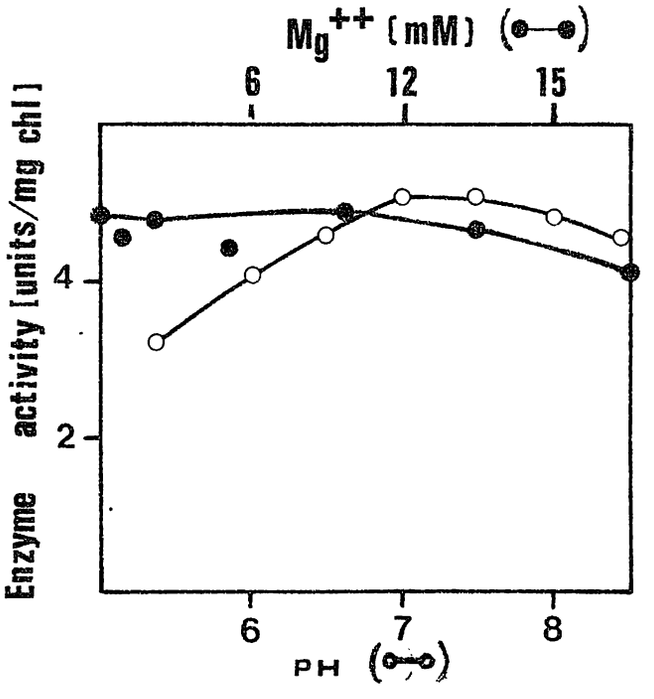
Fig. 5 Uptake of a mixture of ^{14}C-aminoacids (○) into isolated chloroplasts' protein synthesis as CPM of TCA precipitable material (●) and inhibition of this incorporation by 50 g/ml of chloramphenicol (■).

Fig. 6 Effect of chloramphenicol (50 μg/ml) on ferredoxin-GOGAT activity for 2 h. Chloroplasts were preincubated with chloramphenicol in light for one hour. Each point is the mean from four independent curves. Inset is the log of the activity against time.

Fig. 7 Residual activity after precipitation of a constant amount of activity of ferredoxin-GOGAT with antibodies rised against the purified enzyme. Chloroplasts were incubated in light (○), dark (■) and with chloramphenicol (50 μg/ml) (●). Enough antibodies (230 μl) were added to precipitate five units of enzyme at zero time. The same amount of antibodies were added to five units of activity at each point. Due

to the increase in the enzyme volume (five units) the assay was adjusted to 3 ml instead to one with the phosphate buffer. The experiment with chloramphenicol was not followed to 3 h due to the breakage of the chloroplasts in this conditions. Bovine serum albumin was increased to 0.3% in this experiment.


4
2
1
2
3
Time (hs)


Mg++ (mM)
6
12
15
Enzyme activity (units/mg chl)
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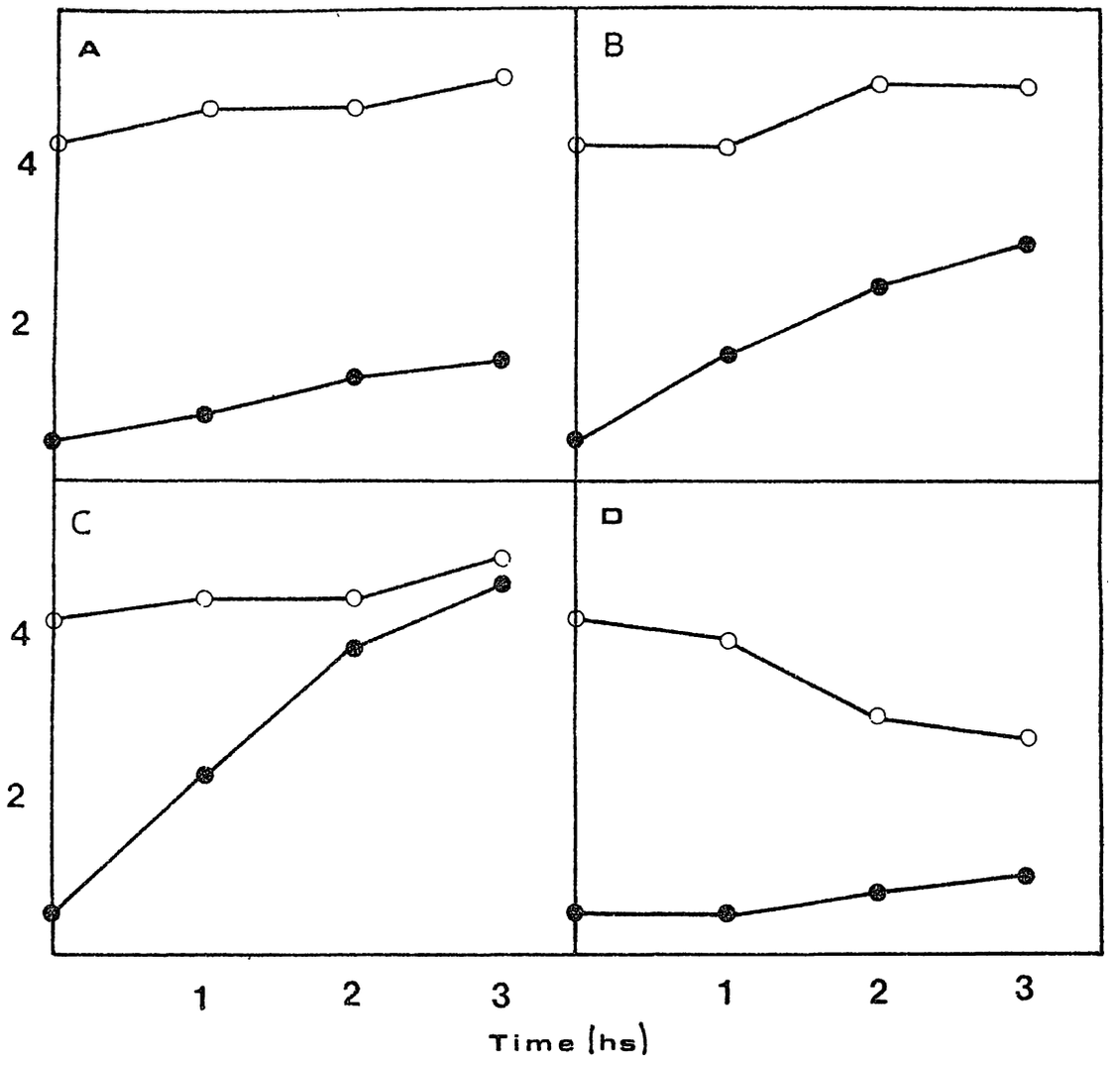
2
6
7
8
PH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C
D
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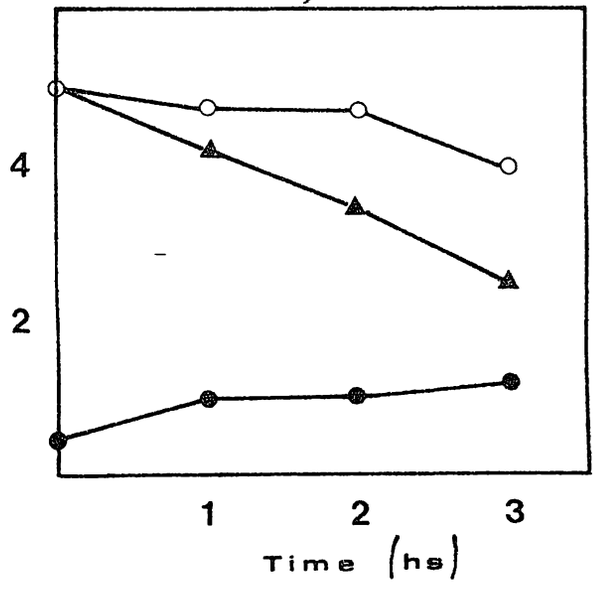
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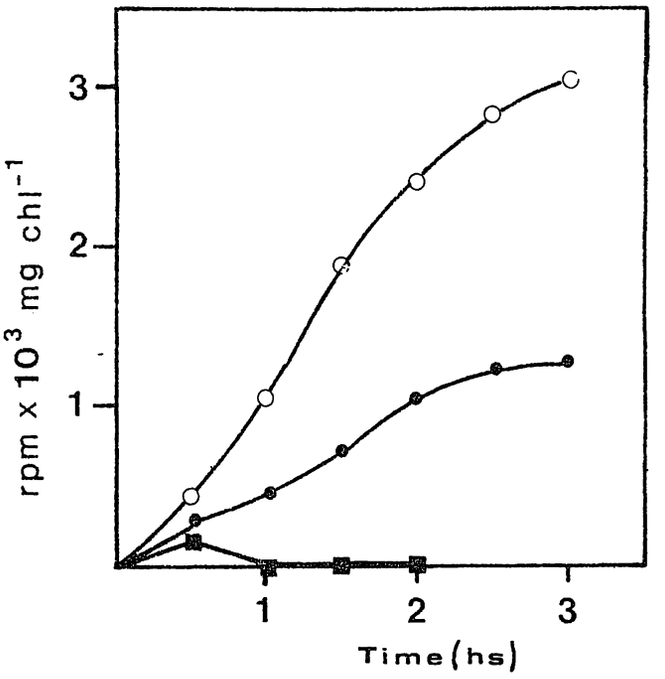
Time (hs)


rpm x 10^3 mg chl^{-1}
3
2
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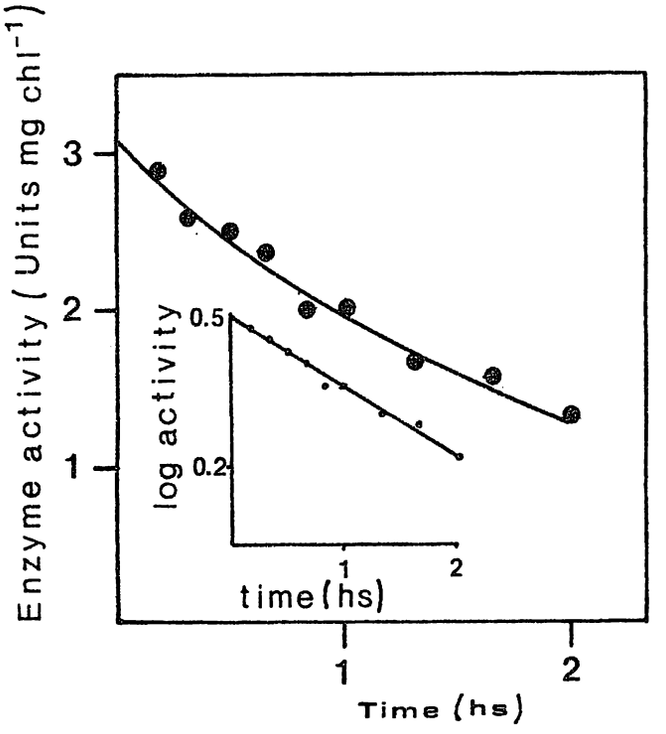
2
3
Time (hs)


Enzyme activity (Units mg chl^{-1})
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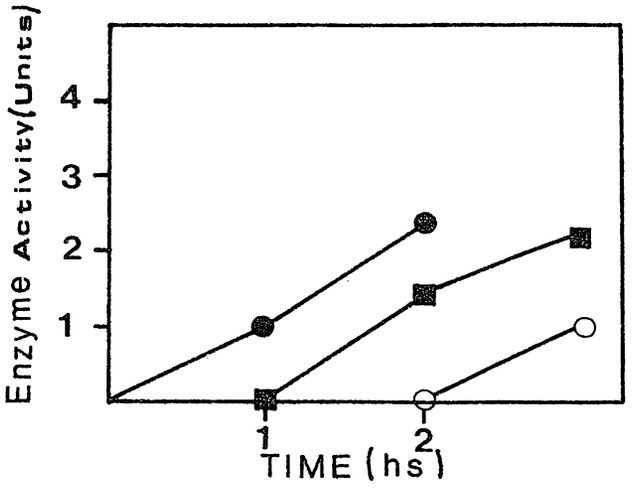
2
1
log activity
0.5
0.2
1
2
time (hs)
1
2
Time (hs)


Enzyme Activity(Units)
4
3
2
1
1
2
TIME (hs)

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