Arch Microbiol (1984) 140:101–106

Archives of
Microbiology

Original papers

Botryococcus braunii carbon/nitrogen metabolism as affected by ammonia addition*

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Abstract. Carbon metabolism in photosynthesizing and respiring cells of *Botryococcus braunii* was radically changed by the presence of 1 mM NH_4Cl in the medium, when the so-called "resting state" previously had been subjected to a nitrogen-deficient medium. Ammonia addition to the algae photosynthesizing with ^{14}C-labelled HCO_3^- almost completely inhibited the synthesis of ^{14}C-labelled botryococcenes and other hexane-extractable compounds, and also inhibited the formation of insoluble compounds; however, it resulted in a large increase in the synthesis of alanine, glutamine, other amino acids, and especially of 5-aminolevulinic acid. Total CO_2 fixation decreased about 60% and O_2 evolution decreased more than 50%.

CO_2 fixation in the dark with ammonia present led to labelled products derived from phosphoenolpyruvate carboxylation, such as glutamine, glutamate, and malate. Respiratory uptake of O_2 increased by about 70%.

The inhibition of terpenoid synthesis and increased synthesis of C_5 amino acids by *Botryococcus* upon ammonia addition indicates 1) a diversion of acetyl coenzyme A from synthetic pathways leading to terpenoids and 2) increased operation of pathways leading to the synthesis of amino acids, especially 5-aminolevulinic acid, a precursor to chlorophyll biosynthesis.

Key words: 5-aminolevulinic acid – Ammonia – *Botryococcus braunii* – Botryococcenes – Carbon – Hydrocarbon – Metabolism – Nitrogen – Resting state – Terpenoids

The oil-producing green alga *Botryococcus braunii* exists in at least two presumably interconvertible states, distinguishable by qualitative hydrocarbon content. Green "active state" colonies produce up to 36% by dry weight of linear olefins (Largeau et al. 1980), mainly dienes and trienes from C_{17} to C_{33} (Gelpi et al. 1968; Knights et al. 1970). The so-called "resting state" colonies, which often appear orange, red, or brown due to accumulated carotenoids, contain mixtures of unusual branched chain olefins (C_nH_{2n-10}, n=30–37) (Wake and Hillen 1981) thought to be of terpenoid origin (Cox et al. 1973). Estimates of the dry weight fraction of botryococcenes from natural populations range from 27 to 86% (Brown et al. 1969; Wake and Hillen 1981).

Little is known about the regulatory mechanisms in this organism controlling the allocation of reduced carbon from photosynthesis to secondary product biosynthesis. Our recent investigations on the flow of carbon from photosynthetic $^{14}CO_2$ reduction into secondary products in this alga revealed patterns of ^{14}C-labelled compounds in the aqueous extracts similar in some respects to those of the widely studied alga, *Chlorella pyrenoidosa*. Of particular interest in *Botryococcus* is the regulation of the supply and utilization of acetyl-CoA, which is the key metabolite used in synthesis of both terpenoids and linear fatty acids and olefins.

Addition of ammonia to *Chlorella pyrenoidosa* has been shown to cause immediate large changes in metabolism (Kanazawa et al. 1970, 1972, 1983). These changes have been interpreted as resulting from stimulation of the reactions mediated by phosphoenolpyruvate carboxylase (PEPC) and pyruvate kinase (PK). Both reactions affect the supply of acetyl-CoA via well-known pathways of intermediary metabolism. In fact, the rate of labelling of lipophylic compounds was increased in photosynthesizing *Chlorella* by ammonia addition. A study was therefore made of changes in *Botryococcus braunii* metabolism following brief nitrogen starvation and then addition of ammonium chloride. The dramatic shifts in carbon metabolism upon addition of ammonia, especially following prior nitrogen starvation, provide possible clues to some of the mechanisms of regulation of carbon compound conversions in this organism.

Materials and methods

Botryococcus culture. Colonies of *Botryococcus braunii* were originally isolated to unialgal culture by Dr. Arthur M. Nonomura from a water lily culturing tank in the Department of Botany greenhouse at the University of California, Berkeley. Cells from this isolate were grown in a medium consisting of (mg l^{-1} of water): $Ca(NO_3)_2 \cdot 4H_2O$ (100), NH_4Cl (26.5), $MgSO_4 \cdot 7H_2O$ (25), K_2HPO_4 (10), H_3BO_3 (0.6), MOPS buffer (3.14), Na_2EDTA (7.7), $ZnCl_2$ (0.624),

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* This work was supported in part by the Office of Energy Research, Office of Basic Energy Sciences, Biological Energy Research Division of the U.S. Department of Energy under Contract No. DE-AC03-76SF00098, in part by a grant from SOHIO, and, in part, by a grant from the Japan-U.S. Cooperative Science Program (The Japan Society for the Promotion of Science, National Science Foundation, Division of International Programs)

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$CuCl_2 \cdot 2H_2O$ (0.268), $NaMoO_4 \cdot 2H_2O$ (0.252), $CoCl_2 \cdot 6H_2O$ (0.42), $FeSO_4 \cdot 7H_2O$ (2.5), and $MnCl_2 \cdot 4H_2O$ (0.36). The last 7 ingredients were added as a 1,000-fold concentrated stock solution which was prepared by boiling the Na_2EDTA in 800 ml H_2O for 5 min and then adding the other salts and bringing the final volume to 1 l. The pH of the final medium was adjusted to 7.2 before autoclaving.

The algal strain was inoculated into the medium and maintained under low illumination (125 $\mu E\ m^{-2}\ s^{-1}$, fluorescent lighting). Two days prior to the experiments, these colony suspensions were diluted with an equal volume of full-strength fresh medium containing 1.0 mM NH_4NO_3, and were grown at 25° C in continuous light (250 $\mu E\ m^{-2}\ s^{-1}$) in an Erlenmeyer flask fitted with cotton-plugged inlet and outlet tubes. Air enriched with 0.3% CO_2 was continously bubbled through the culture via a sintered glass bubbler connected to the inlet tube. For experiments under N-deficient conditions, the colonies were harvested by filtration with a sterile filter unit, resuspended in N-free medium, and incubated further for 2 days under the same conditions. The colonies were again harvested by filtration, washed once with N-free medium, and resuspended in that medium at a 4-fold concentration. Chlorophyll content was about 20 $\mu g\ ml^{-1}$.

Photosynthesis and respiration with labelled carbon dioxide. For each type of sample, 1 ml of the concentrated colony suspension was placed in a 5-ml Erlenmeyer flask. For experiments on photosynthetic carbon fixation, each flask was preilluminated for 20 min (500 $\mu E\ m^{-2}\ s^{-1}$) and then was stoppered with a serum cap. A solution of $NaH^{14}CO_3$ was injected by syringe needle to give a final concentration of 6 mM (40 $\mu Ci\ \mu mol^{-1}$). In one set of flasks, a solution of NH_4Cl was added 5 s before the bicarbonate to a final concentration of 1 mM. At appropriate time intervals, as described in RESULTS, colonies were killed by the addition of 4 ml of hot methanol.

For experiments following changes in metabolite labelling in the dark, colonies were allowed to photosynthesize with the ^{14}C-labelled bicarbonate for 20 min. The flasks were then unstoppered and kept in the dark. Ammonia was added to some flasks after 1 h in the dark. Colonies were killed by addition of hot methanol.

In experiments to determine the products of dark fixation, the colonies were placed in darkened flasks for 20 min before labelled bicarbonate was introduced. After another 20 min in the dark, ammonia was added, and the colonies were subsequently sampled and killed.

Analysis of products. In the killed colony mixtures, the methanol-water mixture had already partially extracted the labelled compounds. Each such mixture was centrifuged and the supernatant fluid was separated from the cell residue. This cell residue was further extracted 3 times with 100% methanol, then once with hexane. Based on preliminary experiments, it was shown that further extraction with water was unnecessary. The combined supernatants from the methanol-water, methanol, and hexane extractions were reduced to dryness in a conical centrifuge tube with a stream of N_2. Then, 2 ml H_2O plus 5 ml hexane were added to each dried extract, the tube contents were well mixed, the layers were separated by centrifugation, and the hexane layer was transferred to another vial. The aqueous mixture was extracted twice with hexane and the hexane extracts were combined and concentrated. A 50 µl sample was removed for determination of radiocarbon by scintillation counting. The remaining hexane extract was used for analysis of hydrocarbon compounds.

A 50 µl sample from the aqueous phase was acidified with acetic acid, dried, and taken up in 0.5 ml H_2O. After addition of 5.5 ml scintillation fluid (Aquashure, New England Nuclear Co, Boston, MA), the total ^{14}C was determined by scintillation counting. A portion of each aqueous sample was analyzed for individual labelled compounds by two-dimensional paper chromatography and radioautography (Pedersen et al. 1966). To aid in subsequent identification of ^{14}C-labelled compounds, solutions containing known amino acids and 5-aminolevulinic acid (5-ALA) were added (about 50 µg of each compound was added per paper).

Chromatograms were developed for 22 h in each solvent (phenol-acetic acid-water and butanol-propionic acid-water; Pedersen et al. 1966). Radioautographs were prepared by exposure of the paper chromatograms to X-ray film for about 1 week. Those with added amino acids were sprayed with ninhydrin solution (0.1% w/v in ethanol) and heated in a film-drying oven for about 5 min. The resulting colored spots on the paper were compared for position and shape with the darkened areas of the film. A spot strongly labelled with ^{14}C (after ammonia addition to the algae) ran close to a position occupied by proline but did not cochromatograph with proline. It did cochromatograph precisely with 5-ALA, which, like proline, gives a yellow color after ninhydrin spraying and heating. The detailed shapes of the darkened area of the film and of the colored area on the paper exactly coincided in all respects. Five such cochromatograms were prepared.

As an additional check on the identity of 5-ALA, the area of the paper containing the radioactive compound was eluted and tested according to the method of Mauzerall and Granick (1956) by condensation with acetyl acetone and colorimetric determination with Ehrlich's reagent. A positive result was obtained which indicated that the compound is an amino-ketone.

HPLC analysis of the botryococcenes. The crude hexane extracts were evaporated under a stream of N_2 to about 0.1 ml, loaded onto a silica gel column (5 mm diameter, 60 mm long) and eluted with hexane. The eluate was analyzed by reverse phase HPLC on two coupled ODS columns (25 cm × 4.6 mm I.D.) using 80:20 methanol:acetonitrile as the mobile phase at a flow rate of 1 ml per min. Eluted compounds were detected by ultraviolet absorption at 215 nm.

Oxygen uptake and evolution measurements. Oxygen evolution or consumption was determined with an oxygen electrode (Rank Brothers Co, England).

Chlorophyll determination. A 5-ml culture sample was filtered and the colonies were rinsed with 1.5 ml of H_2O into a glass homogenizing tube containing 3 ml of acetone and a small amount of alumina. The mixture was homogenized, and the supernatant solution was removed and stored in the dark at −20° C. The pellet was extracted with 4 ml of acetone with stirring for 2 h in the dark at −20° C. The combined extract was adjusted to 10 ml in a 4:1 acetone: water solution and centrifuged at 1,000 × *g* for 10 min.

The absorbance was read at 645 and 663 nm, and the total chlorophyll content was estimated using the equations of Bruinsma (1963).

Results

Hydrocarbons of the Berkeley isolate. The hydrocarbon fraction, which had been previously characterized using GLC and GC-MS (Wolf 1981), was found to be fundamentally the same in this culture when analyzed by the same methods. This fraction consisted almost entirely (>98%) of about 10 C_{30}–C_{34} botryococcenes, which constituted about 25–30% of the total dry weight of the cells.

Nitrogen "starvation" effects. The effects of ammonia addition on *Botryococus* metabolism were in all cases greater when the organism was first subjected to a period of photosynthesis in N-free medium for 2 days. The results described below were obtained from experiments with such N-deprived algae.

Effects of ammonia addition during photosynthesis. Addition of ammonia to "resting-state" *Botryococcus* during photosynthesis resulted in slightly reduced incorporation of $^{14}CO_2$ into some aqueous-extractable compounds. A large decrease was observed in the rates of ^{14}C incorporation into hexane-soluble compounds (including botryococcenes) (Fig. 1) and insoluble compounds (Fig. 2). Consequently, total ^{14}C incorporation decreased to about 70% of the control at 2 min and 40% by 10 min. In separate experiments with an oxygen electrode and somewhat lower effective light intensity, the addition of ammonia decreased the rate of O_2 evolution to less than 50% of the control (Table 1).

Although the total incorporation of ^{14}C into aqueous extractable compounds was not greatly changed, the distribution of label among these compounds was altered significantly (Table 2). At 10 min after ammonia addition, the labelling of sucrose, the predominant labelled compound in the control, was reduced 92%. Labelling of hexose monophosphates (which include fructose 6-phosphate, glucose 6-phosphate, and sedoheptulose 7-phosphate) was reduced 93%.

In contrast, the labelling of alanine increased 14-fold with ammonia addition, while the level of labelled glutamine, below limits of detection in the control, became substantial, and labelled glutamate increased about 40%. However, the levels of labelled aspartate, malate, and fumarate, all products of phosphoenolpyruvate carboxylation, dropped substantially, unlike in experiments with *Chlorella* (Kanazawa et al. 1970) where the labelling of these C_4 compounds usually increased after ammonia addition.

The most heavily labelled compound appearing after ammonia addition in *Botryococcus* (accounting for 38% of the incorporated ^{14}C) was located on the chromatogram at a position where the control showed only minor labelling of a compound previously unidentified in our studies. This compound was identified by cochromatography (see Methods) as 5-ALA, which runs on the chromatograms to the inside of a triangle formed by the positions of alanine, proline, and valine.

Effects of ammonia in the dark. Addition of ammonia to N-deprived *Botryococcus* in the dark following a period of photosynthesis with labelled $^{14}CO_2$ caused a decrease in sucrose labelling, large increases in the labelling of alanine, glutamine, and a compound identified as 5-ALA on the basis of chromatographic position, and smaller increases in glutamate, malate, and aspartate labelling (Ta-

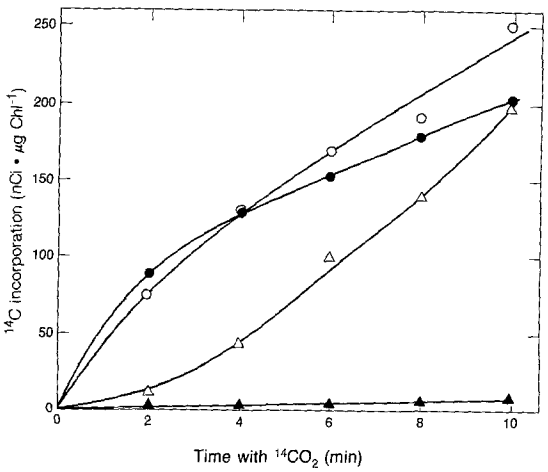

Fig. 1. Effects of ammonia on time course of incorporation of ^{14}C into aqueous- and hexane-extractable compounds. Labelled botryococcenes accounted for about 40% of total labelling of hexane-extractable compounds in all cases. Control: o total aqueous fraction, Δ hexane-extractable. With ammonia addition: ● total aqueous fraction, ▲ hexane extractable

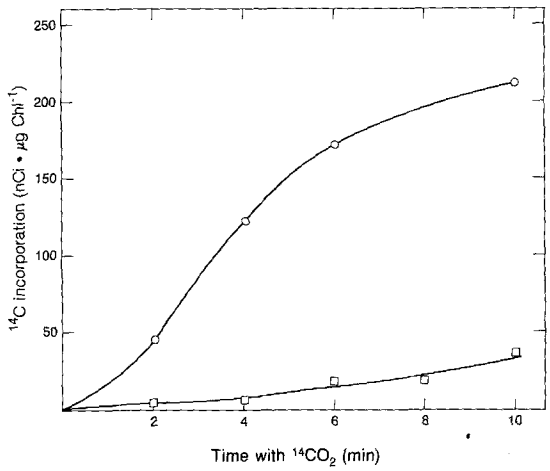

Fig. 2. Effects of ammonia on time course of incorporation of ^{14}C into insoluble compounds. o control; □ plus ammonia

Table 1. Effects of ammonia on the rates of O_2 evolution during photosynthesis and during dark

	A. Control	B. + NH_4Cl (1 mM)	A./B.
	µmol · mg Chl^{-1} h^{-1}		
Photosynthesis			
Exp. 1	+72.9	+34.1	0.47
Exp. 1	+83.7	+40.1	0.48
Respiration (dark)			
Exp. 1	−22.2	−35.7	1.6
Exp. 2	−22.2	−40.4	1.8

For the photosynthesis experiment, the effective light intensity was 500 µE m^{-2} s^{-1}

Table 2. Effect of ammonia on incorporation of ^{14}C into aqueous-extractable metabolites during 10 min photosynthesis with $^{14}CO_2$

Compound	Control	+NH_4Cl (1 mM)
	nCi·μg Chl^{-1}	
Sucrose	77.88	6.19
Hexose monophosphates	12.72	0.88
Malate	10.69	2.97
Fumarate	0.71	0.28
Alanine	2.51	34.90
Aspartate	3.02	1.19
Glutamate	2.79	3.91
Glutamine	–	21.95
5-aminolevulinic acic	–	44.96

Table 3. Effect of added ammonia on distribution of ^{14}C in the dark, following a period of photosynthesis with $^{14}CO_2$. Cells were incubated for one hour in the dark before ammonia addition

Compound	In dark before ammonia addition nCi·μg Chl^{-1}	6 min after ammonia addition nCi·μg Chl^{-1}
Sucrose	22.18	16.18
Alanine	0.23	2.75
Glutamate	0.29	0.73
Malate	0.55	0.82
Aspartate	0.19	0.22
Glutamine	0.14	1.80
5-ALA[a]	0.14	3.49
Unknown cpd.	0.10	2.17

[a] This radioactive spot on the paper chromatogram was tentatively identified as 5-ALA, based on its chromatographic position

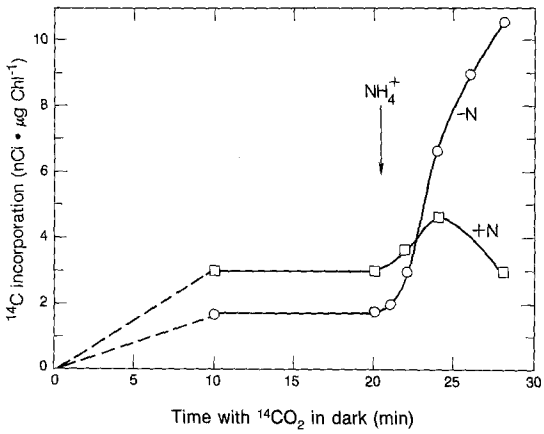

Fig. 3. Effect of ammonia on incorporation of ^{14}C during respiration. Very little ^{14}C was found in hexane-extractable compounds of insoluble material

Table 4. Effects of ammonia added in the dark after 20 min dark fixation of $^{14}CO_2$. Values shown were measured 8 min after ammonia addition

	Percent of total ^{14}C found in major spots on chromatogram
Compound	
Glutamine	46
Glutamate	12
Malate	7
Aspartate	3
5-ALA[a]	20
Unknown compound	13

[a] This radioactive spot on the paper chromatogram was tentatively identified as 5-ALA, based on its chromatographic position

ble 3). Since the actively-turning-over pools of these compounds were probably substantially labelled during the 20 min photosynthesis, these changes in label during the dark indicate ammonia-induced changes in the sizes of these pools in the dark.

To follow just the effects on carboxylation in the dark (principally phosphoenolpyruvate carboxylation), a sample of each type of algae was allowed to photosynthesize with unlabelled carbon dioxide for 20 min. The light was then turned off, the algae darkened, and $^{14}CO_2$ added. When ammonia was added to the medium 20 min later, a large increase in labelled carbon was observed (Fig. 3). Labelled carbon appeared initially in malate with ammonia-treated N-starved algae, but by 6 min, most of the ^{14}C-labelling, as determined by chromatography of the aqueous extract, was in glutamine and two new spots, one of which appeared to be 5-ALA based on chromatographic position. Smaller amounts were found in glutamate, malate, and aspartate (Table 4).

Dark respiration. In a separate experiment, O_2 uptake in the dark was increased about 70% following the addition of ammonia to the respiring algae (Table 1).

Other ions. Addition of 1 mM $CaCl_2$ to the cell suspension caused negligible effects on the rates of O_2 evolution in the light, or of O_2 uptake during respiration. Subsequent addition of 1 mM NH_4Cl caused approximately the same stimulation of both O_2 evolution in the light and O_2 uptake in the dark as seen with NH_4Cl alone.

Discussion

The effects of ammonia on *Botryococcus* seen in this study were in some ways similar to those observed earlier with *Chlorella* and with higher plants. There were some very significant differences, however, that appear to be related to two characteristics of this physiological form of *Botryococcus braunii*. The first is the capability of this alga in the "resting state" to allocate a large proportion of photosynthetically reduced carbon to hydrocarbon biosynthesis. The second difference, observed in this study, is the very rapid flow of carbon into 5-aminolevulinic acid when N-starved cells are given ammonia.

As with *Chlorella*, addition of ammonia in the light decreased the flow of carbon into sucrose and greatly increased the labelling of certain amino acids, especially glutamine and alanine. As was the case with *Chlorella*, these responses indicate a diversion of reduced carbon from sucrose synthesis into synthesis of C_5 amino acids. Since *Botryococcus* converts substantial amounts of reduced carbon into insoluble polysaccharides (Niklas 1976), the observed ammonia-induced inhibition in labelling of insolubles may be due to the large decrease in the pools of labelled hexose monophosphates (Table 2).

An increase in ammonia turnover in cells requires rapid synthesis of glutamine via the glutamine synthase reaction. At the same time, ammonia seems to trigger regulatory mechanisms that stimulate the synthesis of oxoglutarate, thus providing the carbon skeletons for increased C_5 amino acid synthesis (Kanazawa et al. 1970). This synthesis occurs despite the cyclic nature of the glutamate-glutamine-oxoglutarate-glutamate pathway (Keys et al. 1978), and it occurs even in the dark (Kanazawa et al. 1972), when the glutamine-oxoglutarate aminotransferase reaction is presumed not to be operating. This increased flow of carbon into C_5 skeletons requires increases in rates of reactions mediated by PEP carboxylase and pyruvate kinase. These rate increases also can be triggered by the ammonia analog, methylamine, in the absence of strongly accelerated glutamine synthesis (Kanazawa et al. 1983).

In the case of *Botryococcus braunii*, the addition of ammonia in the light apparently also induces the rapid utilization of C_5 carbon compounds for the synthesis of 5-ALA. There is considerable evidence that in higher green plants, the biosynthesis of 5-ALA proceeds via glutamate (Beale et al. 1975; Meller et al. 1975; Harel and Ne'eman 1983). Our in vivo tracer studies do not permit us to distinguish between glutamate and oxoglutarate as the immediate precursor for 5-ALA synthesis, however, since the resulting labelling patterns would be similar in either case. Apparently, following nitrogen starvation of *Botryococcus braunii*, addition of ammonia causes the diversion of C_5 skeletons into 5-ALA synthesis at a rate which competes very favorably with the synthesis of glutamine, even though the latter reaction is required to fix ammonia and thus keep its level from becoming toxic.

Increased C_5 synthesis needs both increased PEP carboxylation to make oxaloacetate and increased conversion of PEP to pyruvate. Subsequent oxidation of pyruvate gives acetyl-CoA for condensation with oxaloacetate to make citrate and eventually oxoglutarate. The great increase in alanine labelling seen in the present study indicates an increased conversion of PEP to pyruvate. The increase in labelling of C_5 compounds in the light and dark and the increased rate of dark ^{14}C uptake are the result of increased PEP carboxylation.

In contrast to the case with *Chlorella pyrenoidosa* (Kanazawa et al. 1970), ammonia addition to this form of *Botryococcus braunii* produces a decrease in labelling of the C_4 compounds aspartate, malate, and fumarate (Table 2). Since the *Botryococcus* "resting state" is synthesizing so much hydrocarbon, there must be a large allocation of reduced carbon to the synthesis of acetyl-CoA. There is evidence for an increased pyruvate kinase activity (see above) with ammonia addition, and consequently an increase in the already-high rate of acetyl-CoA synthesis. Nevertheless, the simultaneous increase in PEP carboxylation must supply enough oxaloacetate to react preferentially with the acetyl CoA, since hydrocarbon biosynthesis is almost non-existent after ammonia addition. Consequently, the rate of synthesis of labelled citrate must be high instead of that of labelled malate and aspartate. This does not result in a large pool of labelled citrate, however. Though labelled citrate was observed in this study, its level was so low that it was not measured. Presumably, the citrate is converted very rapidly to oxoglutarate (also a very small pool), which is then converted to glutamate, glutamine, and 5-ALA, producing the rapid labelling of these compounds.

The small size of the labelled citrate pool at a time of very rapid synthesis of C_5 compounds (5-ALA and glutamine) may be related to the observed increase in the respiratory rate (Table 1). Electron transport in the mitochondria provides for the removal of electrons during the oxidation of citrate to oxoglutarate. The stimulation of respiration observed with *Botryococcus* and also *Chlorella* suggests either an uncoupling of oxidative phosphorylation, or an increased utilization of ATP for energy-consuming reactions. A previous study of isolated plant leaf mitochondria showed no decrease of coupling in the presence of ammonia (Hammel et al. 1979). Since synthesis of both glutamine and 5-ALA requires an increased supply of ATP, this could be one cause of increased respiration in *Botryococcus*.

These effects, while the consequences of a special set of conditions (N-starvation, followed by addition of 1 mM NH_4Cl), may provide clues to normal physiological regulation of the flow of carbon into end products in this metabolically unusual alga. Nitrogen deprivation in nature may be a fairly common occurrence. The results of this study suggest that such deprivation sensitizes the alga to produce a strong regulatory response upon the appearance of intracellular ammonia, and that this response is directed towards preferential use of reduced carbon for synthesis of amino acids and chlorophyll.

Acknowledgements. The authors wish to thank Dr. Arthur M. Nonomura for providing the strain of *Botryococcus braunii* used in this reseach and Dr. T. O-hama for instruction regarding the Ehrlich's test for an α-aminoketone used in confirming the identity of 5-ALA.

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Received March 2, 1984 / Accepted June 28, 1984