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Dark Hexose Metabolism by Photoautotrophically and Heterotrophically Grown Cells of the Blue-Green Alga (Cyanobacterium) *Nostoc* sp. Strain Mac†

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Photoautotrophically grown cells of the blue-green alga (cyanobacterium) *Nostoc* sp. strain Mac assimilated and oxidized both glucose and fructose in the dark at different rates. The rate of fructose metabolism in these cells could be stimulated by casein hydrolysate, the effect being most pronounced at low sugar concentrations. This stimulation was not seen in cells grown heterotrophically in the dark, suggesting that it is a transitory phenomenon which disappears during the autotrophy-heterotrophy growth transition. The stimulation of fructose assimilation by casein hydrolysate was abolished by chloramphenicol or streptomycin, suggesting there are rate-limiting steps in protein biosynthesis in the dark that ultimately lead to inhibition of fructose uptake. Glucose metabolism did not show these phenomena, indicating there are differences in the metabolism of the two sugars.

Much of our information bearing on the heterotrophic metabolism of the blue-green algae (cyanobacteria) is based on the incorporation of radioactive compounds by organisms grown photoautotrophically in the light, which showed no heterotrophic growth potential on the substrates being tested (3, 5, 7, 11, 12, 19). In recent years, although several strains of blue-green algae have been shown unequivocally to be capable of heterotrophic growth in the dark on various hexoses (4, 6, 18, 21, 22, 25), very few studies have been performed with cell material actually grown in this way.

There is evidence that *Chlorogloea fritschii* (4) and *Plectonema boryanum* (17) adapt to heterotrophic growth over a period of time in the dark. These data, along with short-term labeling studies with the heterotrophic strains *Aphanocapsa* sp. strain 6714 (13) and *C. fritschii* (9), indicate that modifications occur in the dark metabolism of these algae brought about by the changeover in growth conditions.

In this paper information is presented about the rates of metabolism of glucose and fructose by the blue-green alga *Nostoc* sp. strain Mac, which is capable of heterotrophic growth in the dark on both sugars (6). The data show that there are fundamental differences between the dark metabolism of these sugars in photoautotrophically and heterotrophically grown cells and that adaptation occurs during the autotrophy-heterotrophy growth transition.

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MATERIALS AND METHODS

Organism. *Nostoc* sp. strain Mac is a filamentous blue-green alga isolated from the coralloid roots of the cycad *Macrozamia lucida* (2) and maintained in this laboratory in the light on slants of medium Cg10 (20), solidified with 1% agar (Difco, 0140).

Photoautotrophic growth conditions. The alga was grown in Pyrex test tubes (175 by 22 mm) in 20 ml of medium Cg10 at 39 ± 0.1°C. Air enriched with 1 ± 0.1% (vol/vol) CO_2 was bubbled through the tubes at a rate of 5 to 6 ml/min, and illumination was provided by two F48T12/CW/XHO fluorescent lamps placed 10 cm from the growth tube positions on either side of the bath. The method is essentially a modification of that of Myers (10). The alga was grown from a slant to an optical density of 0.16 to 0.20, equivalent to 0.07 to 0.09 mg (dry weight) per ml, and 0.1-ml samples of this suspension were used as the inocula to obtain the experimental material.

Heterotrophic growth conditions. The conditions for heterotrophic growth were similar to those described above except that the bath was darkened with black cloth and placed in a dark room. The substrates α-D-(+)-glucose and β-D-(−)-fructose were dissolved in distilled water without heating, filter sterilized through 25-mm-diameter Nucleopore filters (0.45-μm pore size), and added to the growth tubes to give the required concentration. Casein hydrolysate (Matheson, Coleman & Bell, E. Rutherford, N.J.) was stirred in distilled water for several hours, warmed gently to complete the solution, neutralized, and then filter sterilized prior to addition. The inocula for the

heterotrophically grown cells were grown photoautotrophically as described above.

Measurement of growth. Growth was routinely followed turbidimetrically by optical density using a model 402-E Lumetron colorimeter equipped with a red glass 660-nm filter. For dry weight determinations, samples of cultures were filtered through 47-mm-diameter (0.4-μm pore size) Nucleopore filters which had been preweighed to a constant weight by storing over P_2O_5 under vacuum in a desiccator. The algal samples were rinsed dropwise with 5 ml of distilled water, dried at 50°C over P_2O_5 in a vacuum oven for 2 days, and weighed to a constant weight ± 0.05 mg. Under both photoautotrophic and heterotrophic growth conditions, an algal suspension of optical density = 1.0 was equivalent to 0.40 to 0.43 mg (dry weight) per ml. In all experiments described, cells were used which had been growing exponentially under the respective conditions.

Measurement of the dark assimilation of [^{14}C]glucose and [^{14}C]fructose. *Nostoc* sp. strain Mac was grown photoautotrophically or heterotrophically as described above to an optical density equivalent to 0.35 to 0.40 mg (dry weight) per ml. The cells were transferred aseptically to centrifuge tubes and harvested in a Lourdes centrifuge at 7,000 rpm for 10 min. The cells were washed, recentrifuged, and then suspended in 25-ml samples of fresh Cg10 medium to an optical density of 0.4 (0.17 mg [dry weight] per ml). Photoautotrophically grown cells were equilibrated in the light for 1 h and then placed in the dark. In the case of heterotrophically grown cells, all manipulations were done with the laboratory lights off. The cells, after resuspension, were placed in the dark and allowed to reequilibrate. Both types of culture were kept at 39°C and continuously bubbled with air enriched with 1% CO_2. [*U*-^{14}C]glucose and [*U*-^{14}C]fructose (Amersham/Searle Corp., Arlington Heights, Ill.; specific activities, 327 mCi/mmol and 316 mCi/mmol, respectively) were added to the unlabeled stock solutions to give 0.0015 to 0.003 μCi/μmol. A 0.5-ml sample was added to each of the tubes of algal suspension in the dark. Aliquots of 2 ml were removed at 30-min intervals, filtered through 25-mm-diameter, 0.45-μm-pore size Metricel GA-6 filters (Gelman Instrument Co., Ann Arbor, Mich.), and then washed dropwise with 5 ml of ice-cold Cg10 medium containing unlabeled glucose or fructose at the concentration being used in the experiment. The filters were dried and placed in scintillation vials, and the algal material was solubilized overnight in 1 ml of toluene-Soluene 350 (Packard Instrument Co., Downers Grove, Ill.), 3:1. A 5-ml volume of Insta-Fluor scintillation cocktail (Packard Instrument Co.) was added, and the samples were counted on a Packard liquid scintillation spectrometer model 3225.

Carbon dioxide production from radioactive glucose and fructose. The procedure of harvesting and resuspending the cells was the same as described above. The cells were placed in a closed, all-glass test tube system such that the gases bubbled through the algal suspension were subsequently bubbled through a CO_2 trap consisting of 1 ml of Carbo-Sorb (Packard Instrument Co.) in a 7-ml Vacutainer tube (Becton-Dickinson, Rutherford, N.J.). The capacity of this solution was sufficient to hold all of the CO_2 from the enriched air plus that which was respired. The trapping tubes were replaced every 30 min, and the Carbo-Sorb was transferred to scintillation vials along with 5 ml of Insta-Fluor for counting.

Respiration measurements (O_2 uptake). Measurements of oxygen uptake were made using a YSI (model no. 5331) Clark-type oxygen electrode. Samples (1.9 ml) of algal suspensions corresponding to 0.16 to 0.20 mg (dry weight) per ml were added to the chamber (39°C) in darkness and bubbled for 2 min with 1% (vol/vol) CO_2 in air. The chamber was stoppered, and the rate of oxygen consumption was measured as the change in electrode current detected on a Keithley 150 A microvolt ammeter and recorded on a Heathkit recorder.

RESULTS

Assimilation of fructose by photoautotrophically grown cells. Photoautotrophically grown cells of *Nostoc* sp. strain Mac can immediately assimilate fructose in the dark (Fig. 1a). The initial rates were linear and concentration dependent; however, in all cases these rates were maintained only for a few hours. Indeed, at concentrations of 5.6 mM and less the rates decreased almost to zero after 3 h of darkness (see also Fig. 4a). In contrast, the addition of casein hydrolysate (1 g/liter, final concentration) to a similar algal suspension in the dark caused a stimulation of fructose assimilation which was most pronounced at concentrations of 5.6 mM and less (Fig. 1b). The stimulation ranged from two- to fourfold, was apparent in less than a generation time, and could be maintained for at least 6 h. The fructose carbon assimilation was found in ice-cold 10% trichloroacetic acid-precipitable material (data not shown) and indicated that the sugar, on being taken up by the cells, had been further metabolized into polymeric forms.

This stimulation of the dark metabolism of photoautotrophically grown cells of *Nostoc* sp. strain Mac also was seen in a long-term growth experiment (Fig. 2). The data show that the alga would scarcely grow on concentrations of fructose of 5.6 mM and less with nitrate as the nitrogen source, with generation times exceeding 200 h. However, in the presence of casein hydrolysate the growth rates on these fructose concentrations were stimulated by up to 10-fold to a common generation time of 24 h, which could be maintained until the fructose was exhausted. Such a large rapid stimulation of dark metabolism has not been observed previously in the blue-green algae, and further experiments were done in an attempt to characterize it.

Oxidation of sugars by photoautotrophically grown cells. The data in Fig. 3a show that casein hydrolysate caused almost a three-

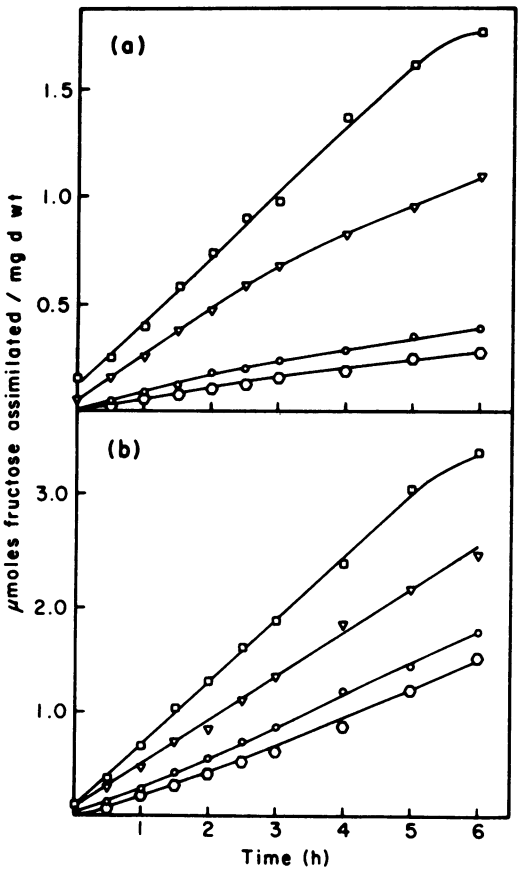

FIG. 1. *(a) Effect of fructose concentration on the rate of assimilation in the dark by photoautotrophically grown cells. [U-^{14}C]fructose was added at time zero; 2-ml samples were taken and treated as described in the text. Fructose concentration: (□) 56 mM; (▽) 28 mM; (○) 5.6 mM; (◯) 1.1 mM. (b) Effect of fructose concentration on the rate of fructose assimilation in the dark by photoautotrophically grown cells in the presence of casein hydrolysate (1 g/liter, final concentration). All symbols represent the same fructose concentrations as in (a). Casein hydrolysate was added with the labeled sugar at time zero in all figures, unless stated otherwise.*

fold increase in the rate of oxidation (CO_2 release) of 5.6 mM fructose, substantiating the increased rate of assimilation (Fig. 1b). However, although the rate of oxidation of 28 mM fructose was higher still, casein hydrolysate did not stimulate this rate to the same extent. In contrast, the rate of glucose oxidation was scarcely affected by casein hydrolysate at either 5.6 mM or 28 mM (Fig. 3b). Essentially casein hydrolysate enabled 5.6 mM fructose to be metabolized at the same rate as 5.6 mM glucose or 28 mM fructose. These data suggest that fructose uptake is not as efficient as glucose and that casein hydrolysate plays a role in overcoming this deficiency. Furthermore, 56 mM glucose only inhibited the stimulated rate of assimilation of 5.6 mM fructose by about 10% (Fig. 4a), and, vice versa, fructose had little effect on the rate or pattern of glucose metabolism (Fig. 4b). This suggests that the two sugars are being metabolized independently of each other in photoautotrophically grown cells and contrasts with the situation with some other heterotrophic microorganisms where glucose quickly switches off fructose transport and metabolism (8).

Effect of inhibitors of protein synthesis. The effect of casein hydrolysate could be abolished by the inhibitors of protein synthesis, chloramphenicol and streptomycin (20 μg/ml, final concentration, a concentration which inhibited incorporation of [^{14}C]leucine by 95% under the same conditions). If chloramphenicol was added at time intervals after casein hydrolysate, then inhibition still occurred but took longer to become effective (Fig. 5). This suggests

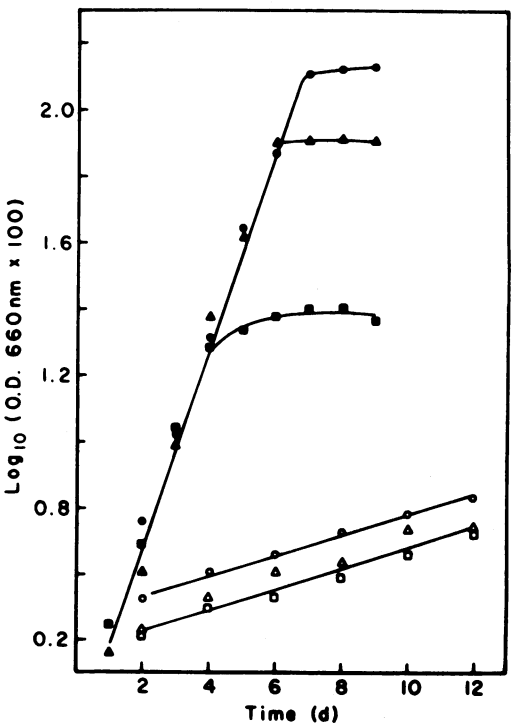

FIG. 2. *Effect of casein hydrolysate (1 g/liter) upon the dark heterotrophic growth rate of Nostoc sp. strain Mac on various fructose concentrations. Growth was measured turbidimetrically at 660 nm as described in the text. Symbols: ○, fructose (5.6 mM); △, fructose (2.8 mM); □, fructose (0.56 mM); ●, fructose (5.6 mM) plus casein hydrolysate; ▲, fructose (2.8 mM) plus casein hydrolysate; ■, fructose (0.56 mM) plus casein hydrolysate.*

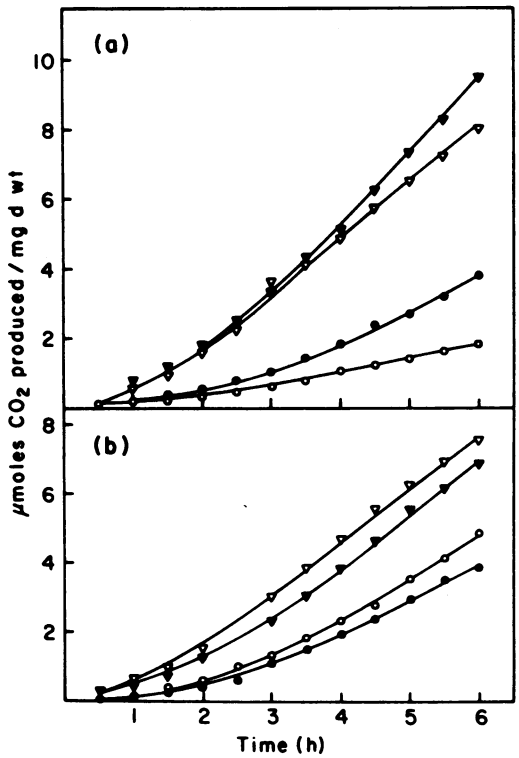

FIG. 3. *(a) Rates of CO_2 production in the dark by photoautotrophically grown cells with 28 mM and 5.6 mM [U-^{14}C]fructose in the presence and absence of casein hydrolysate (1 g/liter). CO_2 was continuously removed from the algal suspension by flushing the closed system with air enriched with 1% (vol/vol) CO_2 and trapped as described in the text. Symbols: ○, fructose (5.6 mM); ●, fructose (5.6 mM) plus casein hydrolysate; ▽, fructose (28 mM); ▼, fructose (28 mM) plus casein hydrolysate. (b) CO_2 production in the dark by photoautotrophically grown cells from 28 mM and 5.6 mM [U-^{14}C]glucose in the presence and absence of casein hydrolysate. Symbols: ○, glucose (5.6 mM); ●, glucose (5.6 mM) plus casein hydrolysate; ▽, glucose (28 mM); ▼, glucose (28 mM) plus casein hydrolysate.*

that continuous de novo protein synthesis is required for the increased rate of assimilation to be maintained. Attempts were made to simulate the effect of casein hydrolysate on fructose assimilation by using individual or mixtures of compounds. Ammonium chloride, glutamine, glutamic acid, asparagine, methionine, and leucine were unsuccessful, as were glucose, malate, succinate, pyruvate, and sodium chloride. Furthermore, a mixture of the 12 predominant amino acids in casein hydrolysate did not stimulate assimilation either. From this it is judged that there may be other unknown factors in this complex mixture which are required along with the amino acids per se to cause the stimulation of fructose metabolism in the dark.

Sugar assimilation and oxidation by heterotrophically grown cells. In contrast to the data obtained with photoautotrophically grown cells of *Nostoc* sp. strain Mac, some differences were seen in fructose metabolism by heterotrophically grown cells. First, the pattern of glucose assimilation was similar to that found in photoautotrophically grown cells (Fig. 6b). Indeed, the steady-state rate of assimilation that

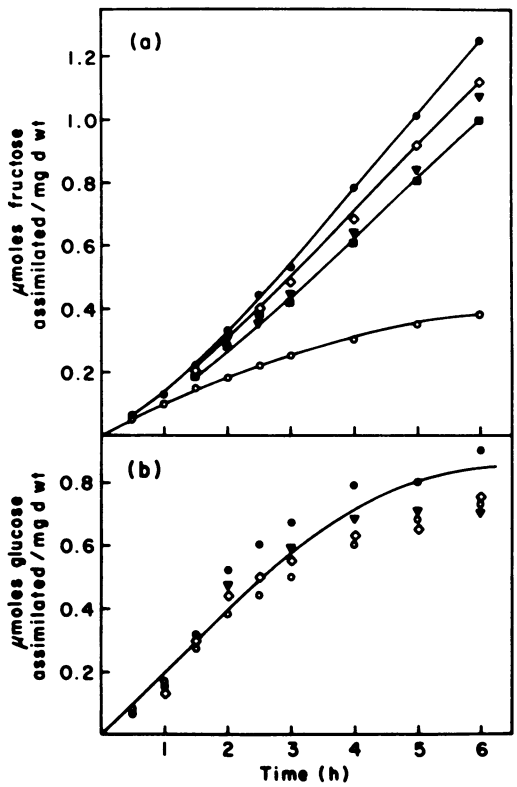

FIG. 4. *(a) Effect of varying concentrations of unlabeled glucose upon the rate of assimilation of [U-^{14}C]fructose (5.6 mM) in the dark in the presence of casein hydrolysate (1 g/liter) by photoautotrophically grown cells. Symbols: ○, fructose (5.6 mM); ●, fructose (5.6 mM) plus casein hydrolysate; ◇, fructose (5.6 mM) plus casein hydrolysate plus glucose (5.6 mM); ▼, fructose (5.6 mM) plus casein hydrolysate plus glucose (28 mM); ■, fructose (5.6 mM) plus casein hydrolysate plus glucose (56 mM). (b) Effect of varying concentrations of unlabeled fructose upon the rate of assimilation of [U-^{14}C]glucose (5.6 mM) in the dark in the presence of casein hydrolysate (1 g/liter) by photoautotrophically grown cells. Symbols: ○, glucose (5.6 mM); ●, glucose (5.6 mM) plus casein hydrolysate; ◇, glucose (5.6 mM) plus casein hydrolysate plus fructose (5.6 mM); ▼, glucose (5.6 mM) plus casein hydrolysate plus fructose (28 mM).*

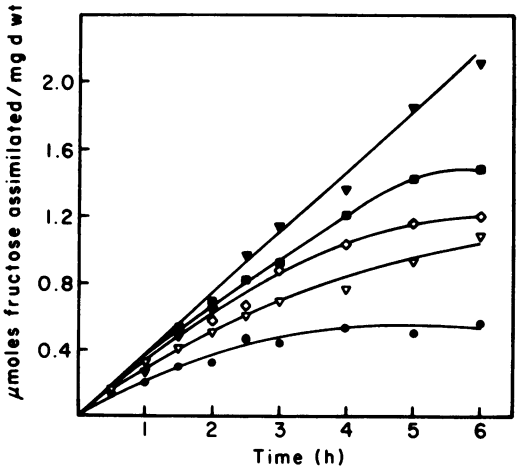

FIG. 5. *Effect of chloramphenicol (20 μg/ml, final concentration) upon the rate of assimilation of [U-^{14}C]fructose (28 mM) in the presence of casein hydrolysate (1 g/liter) by photoautotrophically grown cells. Symbols: ▽, fructose (28 mM); ▼, fructose (28 mM) plus casein hydrolysate added at zero time; ●, fructose (28 mM) plus casein hydrolysate and chloramphenicol added at zero time; ◇, fructose (28 mM) plus casein hydrolysate added at time zero and chloramphenicol added 1 h later; ■, fructose (28 mM) plus casein hydrolysate added at time zero and chloramphenicol added 2 h later.*

was attained 3 h after the sugar was added was sufficient to meet the carbon requirements for growth of cells in the dark on a glucose-nitrate medium. Second, the rate and pattern of glucose assimilation were independent of either the presence or absence of casein hydrolysate or whether the cells had been grown heterotrophically on glucose-nitrate or glucose-casein hydrolysate-containing medium. In the case of fructose, casein hydrolysate did not stimulate the rate of fructose assimilation (Fig. 6a) but simply allowed it to be maintained at its initial rate, which otherwise declined 3 h after fructose addition in the dark. The rates of oxidation of both fructose (Fig. 7a) and glucose (Fig. 7b) were similar in both the presence and absence of casein hydrolysate and were significantly less than the rates obtained with photoautotrophically grown cells (Fig. 3). Furthermore, the O_2 uptake rates of heterotrophically grown cells were substantially lower also (Table 1). Indeed, the amount of both sugars oxidized was independent of whether the organism had been grown on a glucose-nitrate medium with a generation time of 48 h or on a glucose-casein hydrolysate-containing medium with a generation time of 24 h.

DISCUSSION

The stimulation of dark fructose metabolism in photoautotrophically grown cells of *Nostoc* sp. strain Mac by casein hydrolysate was remarkable since it is the first observation of such a large rapid stimulation of dark metabolism in the blue-green algae that was manifested in the growth rate also. At present, the physiological basis for the stimulation is not clearly understood, especially since glucose metabolism was not affected in a similar way. However, since continuous protein synthesis was required to maintain the effect, this suggests that there are rate-limiting steps in protein metabolism in the dark which can be bypassed or stimulated by a factor(s) found in the complex mixture and which profoundly affect fructose metabolism. Moreover, since casein hydrolysate was most effective in stimulating the metabolism of low concentrations of fructose, this suggests that one role, at least, is in alleviating the problems of transporting fructose into the cells.

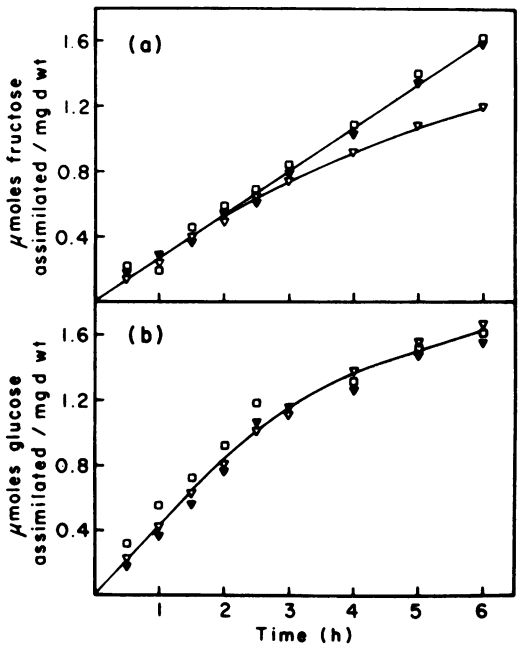

FIG. 6. *(a) Rates of assimilation of [U-^{14}C]fructose (28 mM) in the dark by cells grown heterotrophically on Cg10 medium plus glucose (28 mM) either in the presence or absence of casein hydrolysate (1 g/liter). In both Fig. 6 and 7, the data for fructose or glucose alone (▽) or fructose or glucose plus casein hydrolysate (▼) were obtained from cells grown with nitrate as the nitrogen source; data for fructose or glucose plus casein hydrolysate (□) were obtained from cells grown with casein hydrolysate as an additional nitrogen source. (b) Rates of assimilation of [U-^{14}C] glucose (28 mM) in the dark by cells grown heterotrophically as described above. Curves are labeled as for (a).*

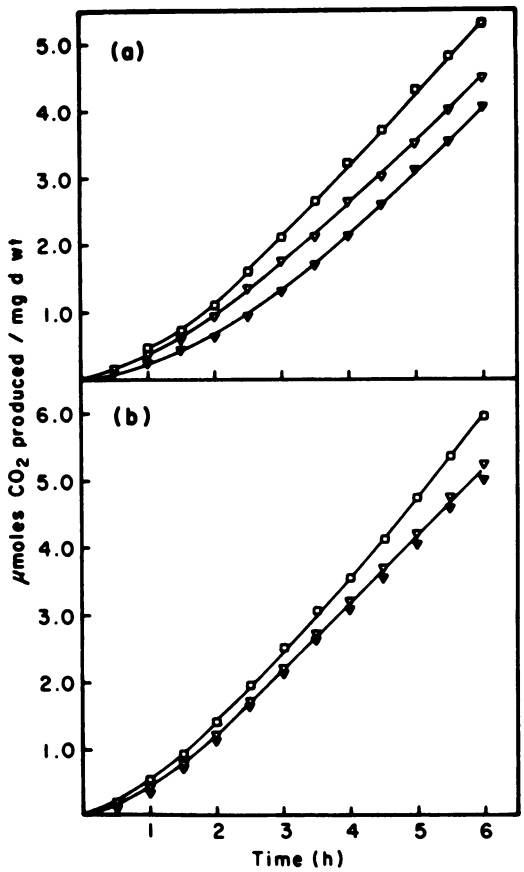

FIG. 7. *(a) Rates of CO_2 production in the dark from [U-^{14}C]fructose (28 mM) by cells grown heterotrophically as described in the legend to Fig. 6. CO_2 was continuously removed from the suspension by flushing the closed system with air enriched with 1% (vol/vol) CO_2 and trapped as described in the text. (b) Rates of CO_2 production in the dark from [U-^{14}C] glucose (28 mM) by cells grown heterotrophically as described above. See legend to Fig. 6 for labeling of curves.*

A stimulation of the growth of blue-green algae by organic carbon and nitrogenous compounds has been observed previously under suboptimum conditions for photoautotrophic growth (i.e., in blue light) (15). These workers found that the growth rates of several blue-green algae could be stimulated by supplementing the growth medium with glucose and/or casein hydrolysate, which indicated that growth of the organisms was limited on either the carbon or nitrogen side of metabolism or even both. Indeed, previous data obtained on the characteristics of heterotrophic growth of *Nostoc* sp. strain Mac suggested that whereas growth on glucose was limited by the rate of nitrogen assimilation, growth on fructose was limited by carbon assimilation (P. J. Bottomley and C. Van Baalen, J. Gen. Microbiol., in press). The labeling data described here confirm this idea. Furthermore, *Nostoc* sp. strain Mac is characterized by having rather high levels of the enzyme glucose dehydrogenase (14, 16), which has been shown to be involved in transport of glucose in *Pseudomonas aeruginosa* when grown under nitrogen-limiting conditions (23, 24). It is therefore plausible to consider this enzyme as playing a key role in the transport of glucose into the cell and accounting in part for the differences in growth on glucose and fructose.

Along with the differences seen in the metabolism of the two sugars by photoautotrophically grown cells, heterotrophically grown cells of *Nostoc* sp. strain Mac showed several differences in their dark metabolism of the sugars, which indicated that adaptations had been made during the autotrophy-heterotrophy growth transition. First, casein hydrolysate did not stimulate fructose assimilation over a concentration range of 56 to 5.6 mM but simply allowed the initial rate of assimilation to remain similar to that of the steady-state rate of glucose assimilation by such cells. In the absence of casein hydrolysate, these rates of fructose assimilation could not be maintained and fell to very low rates indeed. Second, although the rate of assimilation of glucose remained similar to photoautotrophically grown cells, the rate of CO_2 production was substantially less, and this was supported also by lower O_2 uptake values observed for hetero-

TABLE 1. *Rates of dark oxygen uptake by cells of Nostoc sp. strain Mac under various growth conditions*[a]

Growth conditions	Respiratory rate (μmol of O_2 per mg [dry weight] per h)
Photoautotrophically grown:	
Cg10 with 1% CO_2 in air	0.90
Heterotrophically grown:	
Cg10 + glucose (28 mM)	0.38
Cg10 + glucose (28 mM) + casein hydrolysate (1 g/liter)	0.61
Cg10 + fructose (28 mM)	0.33
Cg10 + fructose (28 mM) + casein hydrolysate (1 g/liter)	0.52

[a] *Nostoc* sp. strain Mac was grown at 39°C under the various conditions described above to equivalent densities of about 0.2 mg (dry weight) per ml. Samples (1.9 ml) of cells were carefully transferred in darkness to the oxygen electrode chamber and bubbled with 1% CO_2 in air for 2 min, and the chamber was sealed. The rates of oxygen consumption were monitored for 15 min as described in the text.

trophically grown cells. Indeed, the data indicate that dark glucose metabolism has become more "tightly coupled" during the growth transition, in contrast to fructose, where an imbalance between oxidation and assimilation still exists. These data substantiate earlier findings upon the efficiency of cell synthesis on glucose and fructose reported elsewhere (Bottomley and Van Baalen, in press). Third, the data also show that heterotrophic growth on the sugars did not cause induction of a transport system and thus are in agreement with data obtained on the blue-green alga *Aphanocapsa* sp. 6714 (1) but contrast with the situation in *P. boryanum* where the rate of glucose assimilation increased markedly in cells subjected to heterotrophic growth conditions (17). Certainly further studies are required to learn more about the adaptations to, and control of, heterotrophic growth and metabolism in this predominantly autotrophic group of microorganisms.

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