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Inorganic Carbon Limitation and Chemical Composition of Two Freshwater Green Microalgae†

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Two freshwater chlorophytes, *Chlorella vulgaris* and *Scenedesmus obliquus*, were grown in inorganic carbon-limited continuous cultures in which HCO_3^- was the sole source of inorganic carbon. The response of the steady-state growth rate to the external total inorganic carbon concentration was reasonably well described by the Monod equation; however, the response to the internal nutrient concentration was only moderately well represented by the Droop equation when the internal carbon concentration was defined on a cellular basis. The Droop equation was totally inapplicable when total biomass (dry weight) was used to define internal carbon because the ratio of carbon to dry weight did not vary over the entire growth rate spectrum. In batch cultures, maximum growth rates were achieved at the CO_2 levels present in atmospheric air and at HCO_3^- concentrations of 3 mM. No growth was observed at 100% CO_2. Both nitrogen uptake and chlorophyll synthesis were tightly coupled to carbon assimilation, as indicated by the constant C/N and C/chlorophyll ratios found at all growth rates. The main influence of inorganic carbon limitation appears to be not on the chemical structure of the biomass, but rather on cell size; higher steady-state growth rates lead to bigger cells.

Variations in the chemical composition of phytoplankton are tightly coupled to changes in growth rate (18, 42; J. C. Goldman, *in* P. G. Falkowski, ed., *Primary Productivity in the Sea*, in press). To a large degree, this growth rate dependence provides a good description of the nutritional state of a cell population in response to different degrees of nutrient limitation (39; Goldman, in press). For example, significant variations in the cell quota (Q) (cellular concentration of a limiting nutrient) for either phosphorus or nitrogen occur when the respective nutrient is limiting in continuous culture and the dilution rate (≃growth rate) is varied (8, 12, 20, 39). Droop (7) has demonstrated that Q is related to growth rate by a rectangular hyperbolic equation of the following form: $\mu = \bar{\mu}\ (1 - k_Q Q^{-1})$ (equation 1), where μ is the specific growth rate, $\bar{\mu}$ is the specific growth rate for which Q is infinite, and k_Q is the minimum concentration of limiting nutrient required before growth can proceed. This equation is empirical, and its utility is related to which limiting nutrient is being considered (17). For nutrients that constitute a small fraction of total cellular material, such as PO_4^{3-} and vitamin B_{12}, the ratio of k_Q to Q_M (Q_M is the upper boundary of Q, which is associated with the true maximum growth rate [$\hat{\mu}$]) is very small (e.g., <0.1), indicating a large variation in Q for $0 < \mu \leq \hat{\mu}$ (17). In such cases, according to equation 1, $\hat{\mu} \simeq \bar{\mu}$. When nitrogen, which constitutes ~5 to 10% of the total cellular biomass, is the limiting nutrient, the variability in Q is more restricted, and the ratio of k_Q to Q_M is ~0.2, so that $\hat{\mu} \simeq 0.8\ \bar{\mu}$ (17, 20). Under these conditions, the applicability of equation 1 is restricted, and there is no substitute for determining $\hat{\mu}$ and Q_M experimentally.

To date, virtually no information is available concerning the degree of cellular carbon variation in phytoplankton when inorganic carbon is the limiting nutrient. However, Goldman et al. (19) and Pipes (36) did observe that the yield coefficient on a weight basis (cellular dry weight per unit of cellular organic carbon) for several freshwater green algae was invariant over the entire growth rate spectrum in inorganic carbon-limited continuous cultures. However, cell numbers were not measured, and thus Q values for carbon were unavailable.

Moreover, Goldman et al. (19) demonstrated that at a steady state under inorganic carbon limitation μ was related to the residual total inorganic carbon concentration (C_{T_1}) (sum of concentrations of CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}) according to the Monod equation: $\mu = \hat{\mu} C_{T_1} (K_s + C_{T_1})^{-1}$ (equation 2), where K_s is the half-saturation coefficient (the concentration of

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limiting nutrient for which $\mu = \hat{\mu}/2$). In addition, K_s was found to be a function of culture pH. For microalgae it has been difficult to demonstrate relationships between μ and residual (external) limiting nutrients because K_s values for the common nutrients studied (e.g., nitrogen, phosphorus) are typically below levels of detectability, even though the Droop (internal nutrient) and Monod (external nutrient) equations are compatible at steady state (4, 6, 12). However, Brown and Button (3) were able to measure residual phosphorus levels in the nanomolar range and found a linear relationship between μ and residual phosphorus levels for steady-state growth of the freshwater chlorophyte *Selenastrum capricornutum*. Moreover, they found that a threshold level of 10 nM phosphorus was required before growth could proceed and concluded that the Monod relationship did not describe phosphorus-limited growth well for this alga. Still, the usefulness of the Monod relationship is that it provides a reasonable description of the affinity of a particular organism for a particular limited nutrient. Like the Droop equation, it is purely empirical, but because of its simplicity and the usefulness of K_s, it has popular appeal (12).

In this study, we expanded on the previous study of Goldman et al. (19) and examined the utility of the Droop and Monod equations for inorganic carbon-limited growth of two freshwater green microalgae, *Chlorella vulgaris* and *Scenedesmus obliquus*, in continuous cultures. In addition, we studied how the chemical compositions of these algae varied with growth rate under inorganic carbon limitation.

MATERIALS AND METHODS

The continuous culture apparatus (a bank of eight 0.5-liter cultures), the culturing protocols, and the experimental analyses were virtually identical to those described previously (17, 20). We used continuous lighting (2,093 J of visible light per m^2 per min), temperature control (20°C), and mixing with magnetic bar stirring in the continuous culture experiments. Aeration with mixtures of 100% CO_2 and laboratory air at several bubble rates was used only in some of the batch experiments to determine $\hat{\mu}$. CO_2 from a gas cylinder and laboratory air were first mixed in the desired proportions in a two-gas proportioner. The specific gas bubbling rate (gas bubbling rate per culture volume) was set by passing the gas mixture through a flow meter-regulator before it entered the bottom of the culture in these experiments. In all other experiments gas bubbling was not employed, and HCO_3^- was the sole source of inorganic carbon. The freshwater chlorophytes *C. vulgaris* and *S. obliquus* were obtained from the laboratory of M. Gibbs, Brandeis University.

The freshwater medium was similar to the medium used previously (19) and contained 2.0 mM NH_4Cl, 0.4 mM $MgCl_2$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM $CaCl_2 \cdot 2H_2O$, 0.04 mM H_3BO_4, and trace metals in a twofold dilution of *f* medium (23). The medium for the continuous culture experiments was buffered with 10 mM phosphate buffer consisting of equimolar concentrations of K_2HPO_4 and KH_2PO_4; this resulted in a pH of 7.1 to 7.2. The concentration of total inorganic carbon in the medium (C_{T_0}) was 10 mg of C per liter and was supplied by a mixture of $NaHCO_3$ and $NaCO_3$. For the batch studies the ratio of di-PO_4^{3-} to mono-PO_4^{3-} in 25 mM buffer was varied, depending on the amount of CO_2 in the gas mixture. Up to 10 mM HCO_3^- was added in some of these batch experiments. The medium was dispensed into the continuous cultures via a multichannel peristaltic pump (Harvard 1203). All tubing was glass, except for small sections of silicone which were inserted through the pumps.

Chemical analyses for C_{T_0} and C_{T_1} were performed with a Dohrmann DC-54 Ultra-Low Total Carbon Analyzer modified for inorganic carbon analyses as described by Goldman (13). This instrument has a precision of ±10 μg of C per liter (or ±2%) and a detection limit of ~50 μg of C per liter. Particulate carbon and nitrogen were measured with a Perkin-Elmer model 240 elemental analyzer. Cells were counted with a Spencer Bright-line hemacytometer. Dry weights were determined for 100-ml samples retained on precombusted glass fiber filters and combusted at 500 to 550°C for >4 h. Chlorophyll *a* levels were measured on acetone-extracted samples by fluorometry, using the method of Strickland and Parsons (51). Typically, samples were extracted overnight. Culture and medium pH values were measured with a combination probe mounted on a Corning 110 meter. All measurements were made directly on culture samples at steady state, which was defined as the time when culture absorbance at 600 nm, as measured with a Bausch & Lomb Spectronic 88 spectrophotometer, did not vary more than ±10% for at least 2 consecutive days. The cultures were not axenic for the reasons cited previously (12).

$\hat{\mu}$ was estimated both by the cell washout technique (12) and by the enriched culture batch technique (20). Batch experiments were performed by using either bubbled gas or HCO_3^- as the source of inorganic carbon. The following three concentrations of HCO_3^- were used: 3, 6, and 10 mM. Gas mixtures included air (0.036% CO_2) at three bubbling rates (25, 50, and 75 h^{-1}) and 1, 5, and 100% CO_2 in air at a constant bubbling rate of 50 h^{-1}. $\hat{\mu}$ was determined for each experiment by a linear regression analysis of the plot of the natural log of the cell count versus time. In each experiment several measurements were made during exponential growth for cellular carbon (Q_{cM}) and nitrogen (Q_{nM}). The inocula for the batch cultures were taken from continuous cultures at steady state to give initial cell numbers of 0.1×10^5 to 0.3×10^5 cells per ml.

The kinetic coefficients $\bar{\mu}$ and k_Q were determined from regression analyses of the linearized version of equation 1, as follows: $Y = Y_Q(1-\mu\bar{\mu}^{-1})$ (equation 3), where Y is the cellular yield coefficient and Y_Q is the

maximum cellular yield coefficient (k_Q^{-1}) (7). K_s and $\hat{\mu}$ were determined from regression analyses of the linearized version of equation 2, as follows: $C_{T_1} = (C_{T_1}\mu^{-1})\hat{\mu} - K_s$ (equation 4) (19). The ratio of $\hat{\mu}$ to $\bar{\mu}$ was determined from linear regression analyses by using equations 3 and 4 or by inserting the ratio of k_Q to Q_{cM} into the limiting expression of equation 1, as follows: $\hat{\mu} = \bar{\mu}(1 - k_Q \cdot Q_{cM}^{-1})$ (equation 5) (17). The value of Q_{cM} in this case was determined experimentally. A total of 46 steady-state measurements were made for *C. vulgaris* in the growth rate range 0.17 to 2.05 day^{-1}, and 33 measurements were made for *S. obliquus* in the growth rate range 0.17 to 1.56 day^{-1}.

RESULTS

$\hat{\mu}$. Estimates of $\hat{\mu}$ as determined by the washout technique were 1.59 ± 0.028 day^{-1} (mean ± standard deviation) for *S. obliquus* and 2.11 ± 0.036 day^{-1} for *C. vulgaris*. For *C. vulgaris* the values of $\hat{\mu}$ as determined by the batch technique, regardless of whether HCO_3^- (3 to 10 mM) or bubbled CO_2 (0.036 to 1%) was the inorganic carbon source, were comparable to the values obtained by the washout technique, averaging 2.02 ± 0.052 day^{-1} (Table 1). With 5% CO_2 $\hat{\mu}$ was diminished considerably (0.97 day^{-1}), and with 100% CO_2 no growth was observed. On the other hand, for *S. obliquus*, $\hat{\mu}$ as determined by the batch technique (1.56 ± 0.113 day^{-1}) was comparable to $\hat{\mu}$ as determined by the washout method when bubbled CO_2 was the inorganic carbon source at any CO_2 level (except 100% CO_2, at which no growth occurred). Moreover, increasing the concentration of HCO_3^- beyond 3 mM ($\hat{\mu}$ = 1.67 day^{-1}) led to significant reductions in $\hat{\mu}$ (down to 1.16 day^{-1} with 10 mM HCO_3^-) (Table 2). There was no effect of bubble rate on $\hat{\mu}$ for either species when air was the inorganic carbon source. Culture pH values varied between 6.8 and 7.7, with the highest values occurring in the 10 mM HCO_3^- experiments (Tables 1 and 2).

K_s values. The response of μ to the external (residual) inorganic carbon concentration (C_{T_1}) was described well by equation 4, leading to K_s values of 0.20 ± 0.027 mg of C per liter (mean ± standard deviation) for *C. vulgaris* ($r > 0.99$, $P < 0.001$) (Fig. 1A) and 0.16 ± 0.052 mg of C per liter for *S. obliquus* ($r > 0.99$, $P < 0.001$) (Fig. 1B).

Cellular carbon variations. The ratio of cellular carbon to dry weight (Q'_c) was invariant with μ for both species; this ratio was 0.46 ± 0.07 (mean ± standard deviation) for *C. vulgaris* (Fig. 2A) and 0.48 ± 0.08 for *S. obliquus* (Fig. 3A). In contrast, the carbon cell quota (Q_c) increased with μ for both species (Fig. 2B and 3B). The kinetic coefficients k_Q and $\bar{\mu}$, as derived from equation 3, were 2.2 ± 0.09 pg of C per cell and 2.32 ± 0.087 day^{-1} for *C. vulgaris* (mean ± standard deviation), respectively ($r = 0.93$, $P < 0.001$); for *S. obliquus* those values were 7.3 ± 0.04 pg of C per cell and 2.44 ± 0.029 day^{-1}, respectively ($r = 0.80$, $P < 0.001$) (Table 3).

Estimates of Q_{cM} by the batch technique and from the experimental data in Fig. 2B and 3B were similar for both species; values for Q_{cM} were 13.6 ± 1.98 pg of C per cell, as determined by the batch technique (Table 1), to ~15 pg of C per cell, as estimated by eye from the data (Fig. 2B) for *C. vulgaris* and 29.9 ± 5.25 pg of C per cell (Table 2), to ~28 pg of C per cell (Fig. 3B) for *S. obliquus*. The resulting ratios of k_Q to Q_{cM} were 0.16 ± 0.049 (mean ± standard deviation) for *C. vulgaris* and 0.24 ± 0.045 for *S. obliquus* (Table 3). The ratio of $\hat{\mu}$ to $\bar{\mu}$, as determined from the summarized experimental data for $\hat{\mu}$ and $\bar{\mu}$, generally was within the 95% confidence limits of the ratio of $\hat{\mu}$ to $\bar{\mu}$ derived from equation 5 with the ratio of k_Q to Q_{cM} inserted (Table 3); these values were 0.87 ± 0.048, as determined from the experimental values for $\hat{\mu}$ and $\bar{\mu}$, versus 0.86 ± 0.049, as deter-

TABLE 1. *Growth and cellular coefficients for C. vulgaris at $\hat{\mu}$* in batch and continuous cultures

Growth mode	Carbon source	Gas bubbling rate (h^{-1})	$\hat{\mu}$ (day^{-1})	Q_{cM} (pg of C per cell)	Q'_c	Q_{nM} (pg of N per cell)	K_s (mg of C per liter)	Culture pH
Continuous	HCO_3^- (1 mM)		2.11 ± 0.036[a]	~15[b]	0.46 ± 0.071[c]	~2.7	0.20 ± 0.027[a]	7.1-7.2
Batch	HCO_3^- (3 mM)		1.94	12.2		2.7		6.9
	HCO_3^- (6 mM)		2.07	14.2		2.8		7.5
	HCO_3^- (10 mM)		2.02	14.7		3.1		7.7
	CO_2 (0.036%)[d]	25	1.97	12.8		2.5		6.8
		50	2.06	14.0		2.3		6.8
		75	2.08	11.5		2.6		6.8
	CO_2 (1%)	50	1.97	16.0		3.3		6.8
	CO_2 (5%)	50	0.97					6.8
	CO_2 (100%)	50	0					6.0

[a] Estimated by a linear regression analysis of the data in Fig. 1A, using equation 4; mean ± standard deviation.
[b] Estimated by eye from the data in Fig. 2B.
[c] Estimated by determination of the mean of the data in Fig. 2A; mean ± standard deviation.
[d] CO_2 was bubbled through the cultures.

TABLE 2. *Growth and cellular coefficients for S. obliquus at $\hat{\mu}$ in batch and continuous cultures*

Growth mode	Carbon source	Gas bubbling rate (h^{-1})	$\hat{\mu}$ (day^{-1})	Q_{cM} (pg of C per cell)	Q'_C	Q_{nm} (pg of N per cell)	K_s (mg of C per liter)	Culture pH
Continuous	HCO_3^- (1 mM)		1.59 ± 0.028[a]	~28[b]	0.48 ± 0.083[c]	~4.7	0.16 ± 0.052[a]	7.1-7.2
Batch	HCO_3^- (3 mM)		1.67	38.6		5.4		6.8
	HCO_3^- (6 mM)		1.31	22.6		3.4		7.5
	HCO_3^- (10 mM)		1.16	20.2		3.0		7.7
	CO_2 (0.036%)[d]	25	1.50	25.2		4.5		6.8
		50	1.53	29.5		4.4		7.0
		75	1.47	27.6		4.8		6.8
	CO_2 (1%)	50	1.77	33.8		5.3		6.8
	CO_2 (5%)	50	1.58	30.2		4.2		6.8
	CO_2 (100%)	50	0					6.0

[a] Estimated by a linear regression analysis of the data in Fig. 1B, using equation 4; mean ± standard deviation.
[b] Estimated by eye from the data in Fig. 3B.
[c] Estimated by determination of the mean from the data in Fig. 3A; mean ± standard deviation.
[d] CO_2 was bubbled through the cultures.

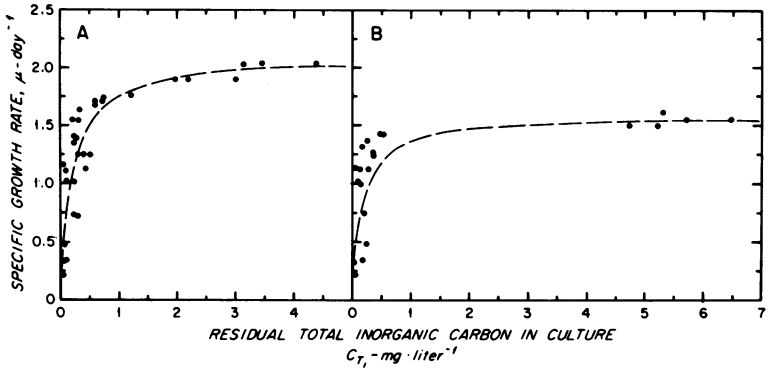

FIG. 1. *Relationship between μ and residual total inorganic carbon in cultures at steady state in inorganic carbon-limited continuous cultures. Curves were determined by linear regression analyses of equation 4 and plots of equation 2. (A) C. vulgaris. (B) S. obliquus.*

mined from equation 5, for *C. vulgaris* and 0.64 ± 0.048 versus 0.76 ± 0.048 for *S. obliquus* (Table 3).

Cellular nitrogen variations. There appeared to be tight coupling between nitrogen assimilation and carbon assimilation at all steady-state growth rates. The cellular ratio of C to N (by weight) was virtually invariant with varying μ values for both *C. vulgaris* (Fig. 4A) and *S. obliquus* (Fig. 4B), ranging between 5 and 6. The maximum nitrogen cellular content (Q_{nM}), as averaged from the batch culture data in Tables 1 and 2, was 2.7 ± 0.40 pg of N per cell (mean ± standard deviation) for *C. vulgaris* and 4.7 ± 0.59 pg of N per cell for *S. obliquus*.

Cellular chlorophyll variations. Cellular chlorophyll content, like carbon and nitrogen contents, increased with increasing μ, ranging from ~0.04 to ~0.25 pg of chlorophyll per cell for *C. vulgarus* (Fig. 5B) and ~0.1 to 0.25 pg of chlorophyll per cell for *S. obliquus* (Fig. 6B) for $0 < \mu < \hat{\mu}$. The ratio of carbon to chlorophyll decreased slightly from ~75 at zero μ to 50 at $\hat{\mu}$ for *C. vulgaris* (Fig. 5A), but generally was invariant at ~100 at all values of μ for *S. obliquus* (Fig. 6A), although there was substantial scatter in the data at $\mu < 0.3$ day^{-1}.

DISCUSSION

Inorganic carbon-limited growth kinetics. Any interpretation of inorganic carbon kinetic data is premised on the knowledge that the actual substrate for assimilation is known or that the rate reactions within the CO_2-HCO_3^--CO_3^{2-} chemical system are all fast enough so that the total flux of inorganic carbon into biomass via photosynthesis is the rate-limiting step (19). Of the several rate reactions in the CO_2-HCO_3^--CO_3^{2-} system, only the following reactions are relatively slow (26): $H_2CO_3 \rightarrow CO_2 + H_2O$ (equation 5) at pH < 8; $HCO_3^- \rightarrow CO_2 + OH^-$ (equation 6) at pH > 10; and both of these reactions at pH 8 to 10. Thus, regardless of whether an alga is an obligate CO_2 user or can assimilate

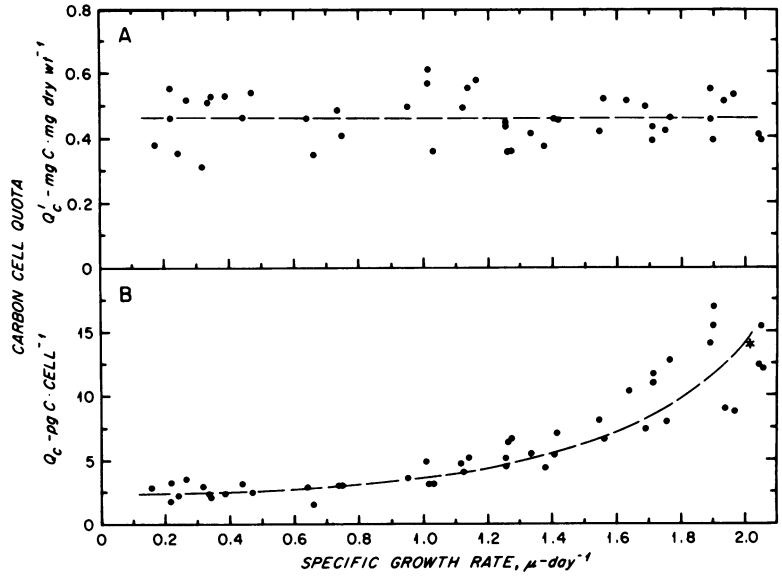

FIG. 2. *Relationship between μ and carbon cell quotas for C. vulgaris in inorganic carbon-limited continuous culture (A) Q'_c, dry weight basis. The curve was based on the mean values of Q'_c. (B) Q_c, cellular basis. The curve was determined by linear regression analyses of equation 3 and plots of equation 1. The asterisk indicates the Q_{cM} from the averaged batch culture data in Table 1.*

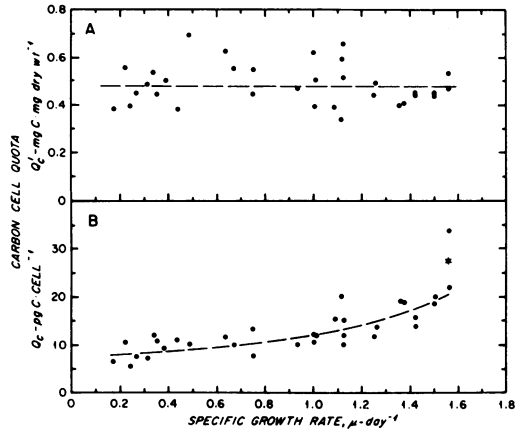

FIG. 3. *Relationship between μ and carbon cell quotas for S. obliquus grown in inorganic carbon-limited continuous cultures. (A) Q'_c, dry weight basis. The curve was based on the mean values of Q_c. (B) Q_c, cellular basis. The curve was determined by regression analyses of equation 3 and plots of equation 1. The asterisk indicates the Q_{cM} from the averaged batch culture data in Table 2.*

HCO_3^- directly, the uptake of any carbon species is indistinguishable from the uptake of the total inorganic carbon pool when the reactions described above are not rate limiting. For example, Goldman et al. (19) demonstrated that for a range of growth rates, algal biomasses, and total inorganic carbon concentrations similar to those used in the current study and for most natural water situations in which the amount of total inorganic carbon present is in excess relative to the demand of phytoplankton, the reactions of equations 5 and 6 generally are not rate limiting; hence, under these conditions it is valid to use C_{T_1} as the substrate in equation 2.

Moreover, when the pH is varied but the chemical reactions in the CO_2-HCO_3^--CO_3^{2-} system remain nonlimiting, it is impossible to determine the form of inorganic carbon used in photosynthesis by comparing K_s values that are based on the relative CO_2 and HCO_3^- concentrations. The two K_s values under these conditions are always related by the equilibrium constants defining the chemical system (16). Lehman (27) carried these arguments further by showing that even when CO_2 was the source of inorganic carbon for photosynthesis, facilitated transport of HCO_3^- across cell membranes supplemented CO_2 transport to maintain high total fluxes of inorganic carbon to the sites of photosynthesis.

As in the previous study of Goldman et al. (19), the relationship between μ and C_{T_1} was described reasonably well by the Monod equation, particularly for *C. vulgaris* (Fig. 1A). For *S. obliquus* in contrast, although K_s and $\hat{\mu}$ were well described by the linear version of equation 2, the shape of the curve in Fig. 1B is probably better described by a first-order–zero-order type of relationship than by a rectangular hyperbola (equation 2). Without a more detailed investigation of this possible effect, which is beyond

the aims of the present study, it is fruitless to speculate further about the true shape of the curves in Fig. 1.

The values of K_s (based on C_{T_1}) for *C. vulgaris* (0.20 ± 0.027 mg of C per liter) and *S. obliquus* (0.16 ± 0.052 mg of C per liter) were of the same magnitude as the K_s values determined for two other chlorophytes, *S. capricornutum* (0.40 mg of C per liter) and *Scenedesmus quadricauda* (0.22 mg of C per liter), grown at pH 7.1 to 7.2 (19). These K_s values are considerably lower than the values found for cultured and natural populations of estuarine and marine phytoplankton measured during short-term ^{14}C incubation studies (5, 28). However, the K_s values determined in these latter experiments are not comparable to those of steady-state continuous culture experiments. In the former case, the values were based on total inorganic carbon uptake over 1- to 2-h incubations, whereas in the latter studies the values were determined as a function of the steady-state growth rate. Photosynthetic rates, particularly when measured over short intervals, are not necessarily coupled to growth rates, mainly because short-term photosynthesis is to a large degree dependent on the physiological state of the cells at the time of sampling (i.e., K_s for uptake is a function of μ) (32).

When K_s values for carbon uptake from different studies are compared, another important consideration is the actual substrate measured to calculate K_s. For example, Markl (29) demonstrated that there was a gradient in the CO_2 concentration between the bulk fluid and the cell surface; thus when *C. vulgaris* was maintained in inorganic carbon-limited turbiostats, the K_s value was ≤1 μg of C per liter, based on CO_2 levels at the cell surface, which were much lower than the CO_2 levels in the bulk liquid (29). By comparison, this K_s value is more than 2 orders of magnitude lower than the K_s values reported in this study, which were based on total inorganic carbon concentrations in the bulk fluid. Thus, it appears that when inorganic carbon is supplied primarily in the gaseous form, the true affinity for inorganic carbon at the cell surface is so high that the main mass transport bottleneck occurs at gas-liquid interfaces. When HCO_3^- is the major source of inorganic carbon, the chemical conversion rates of HCO_3^- to CO_2 for obligate CO_2 users and the efficiency of HCO_3^- transport across cell membranes for species capable of facilitated HCO_3^- transport (27) are the major potential rate bottlenecks. However, it has been demonstrated repeatedly that the enzyme carbonic anhydrase, which catalyzes the reactions of equations 5 and 6, is produced

TABLE 3. *Summary of kinetic data for the two freshwater green algae grown in inorganic carbon-limited continuous cultures*

Species	Growth mode	No. of datum points	$\hat{\mu}$ (day^{-1})	$\bar{\mu}$ (day^{-1})	k_Q (pg of C per cell)	Q_{cM} (pg of C per cell)	Q_{nM} (pg of N per cell)	Ratio of k_Q to Q_{cM}	Ratio of $\hat{\mu}$ to $\bar{\mu}$
C. vulgaris	Continuous	46	2.11 ± 0.036	2.32 ± 0.087[a]	2.2 ± 0.09	15	2.7	0.15	0.92 ± 0.042[b]
	Batch	13[c]	2.02 ± 0.052[d]			13.6 ± 1.98[d]	2.7 ± 0.40[d]	0.16 ± 0.049[b]	0.87 ± 0.048[b]
S. obliquus	Continuous	33	1.59 ± 0.028	2.44 ± 0.029[a]	7.3 ± 0.04	28	4.7	0.26	0.65 ± 0.014[b]
	Batch	12[c]	1.56 ± 0.113[d]			29.9 ± 5.25[d]	4.7 ± 0.59[d]	0.24 ± 0.045[b]	0.64 ± 0.048[b]

[a] Estimated by a regression analysis of the data in Fig. 2B and 3B, using equation 3; mean ± standard deviation.
[b] Mean ± standard deviation. Standard deviations were determined by propagation of errors analyses.
[c] Number of datum points from batch experiments of cellular carbon and nitrogen.
[d] Estimated from mean analyses of data in Tables 1 and 2; mean ± standard deviations.

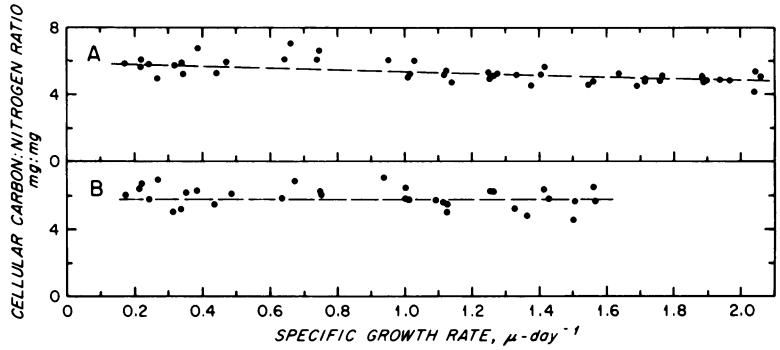

FIG. 4. *Relationship between μ and cellular carbon-nitrogen ratios in inorganic carbon-limited continuous cultures. (A) C. vulgaris. (B) S. obliquus. Lines were drawn by eye and are meant to demonstrate trends only.*

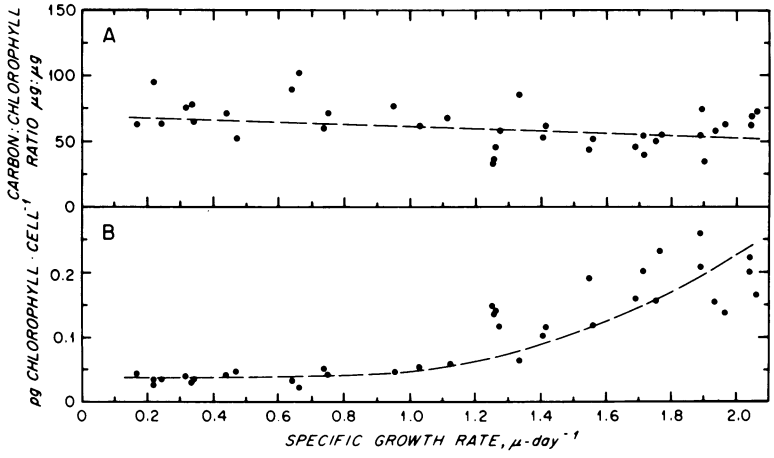

FIG. 5. *Relationship between μ and cellular chlorophyll levels in inorganic carbon-limited continuous cultures of C. vulgaris. (A) Ratio of carbon to chlorophyll. (B) Cellular chlorophyll content. Lines were drawn by eye and are meant to demonstrate trends only.*

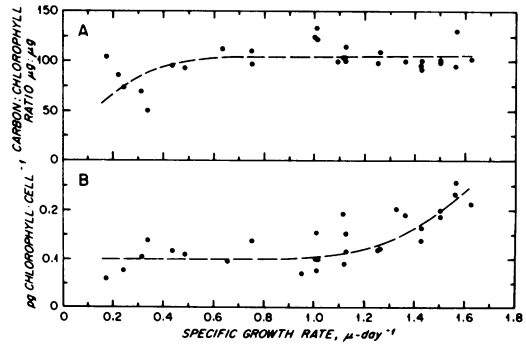

FIG. 6. *Relationship between μ and cellular chlorophyll levels in inorganic carbon-limited continuous cultures of S. obliquus. (A) Ratio of carbon to chlorophyll. (B) Cellular chlorophyll content. Lines were drawn by eye and are meant to demonstrate trends only.*

when cells are grown in low CO_2 environments (1, 11, 21, 22, 25, 33); this provides additional, albeit indirect, evidence that microalgae have very high affinities for inorganic carbon. Therefore, it is virtually impossible to distinguish between uptake of a particular form of inorganic carbon and the response to the entire carbon pool without rapid kinetic experiments, such as

those used by Lehman (27) and Sikes et al. (44).

Sources of inorganic carbon and $\hat{\mu}$. The ability of *C. vulgaris* or *S. obliquus* to grow at maximum rates in batch culture at inorganic carbon concentrations as low as 0.036% CO_2 (Tables 1 and 2) appears to be a common characteristic of many freshwater and marine algae (1, 38, 45, 52) and is another indication of the remarkable affinity which these organisms have for inorganic carbon. There also is general agreement that maximum photosynthetic rates of species such as *Chlorella* spp. and *Scenedesmus* spp. can be sustained with similar and even lower CO_2 concentrations (2, 10, 47, 48).

However, the percentage of CO_2 in the air supplied to a culture is a relatively meaningless term in trying to ascertain the amount of CO_2 required for maximum photosynthesis if no accounting is made of the concentration of the CO_2 in solution which is really available to the algae (31). As demonstrated by Markl (29), this concentration is a function of the sparging rate, the degree of turbulence, and the combined effect of these processes on the CO_2 tension at the cell surface, where the demand for inorganic carbon occurs; for example, with optimum turbulence maximum phytosynthetic rates were attained when the percent CO_2 at the cell surface was 0.0005% (29). The lowest sparging rate (gas bubbling rate = 25 h^{-1}) used in the current study clearly was high enough to prevent any mass transport limitations.

The apparent toxic effect of 100% CO_2 on both species has been observed previously (46), although no satisfactory explanation exists for this phenomenon. The decrease in $\hat{\mu}$ at 5% CO_2 in air observed for *C. vulgaris* (Table 1) is not substantiated by similar data in the literature, as 5% CO_2 has been used commonly to prevent carbon limitation in *Chlorella* and other algal cultures (25, 49). Possibly a lack of conditioning at this CO_2 level led to the apparent reduction in $\hat{\mu}$ (49). Likewise, the decrease in $\hat{\mu}$ for *S. obliquus* with increasing HCO_3^- concentrations greater than 3 mM is difficult to explain. Osterlind (34) found a decrease in μ with increasing HCO_3^- concentrations and concomitant increasing pH values, which he attributed to CO_3^{2-} toxicity. In our cultures the pH rose only slightly from 6.8 at 3 mM HCO_3^- to 7.7 at 10 mM HCO_3^- (Table 2), so that CO_3^{2-} levels were always minimal. Pratt (37) observed that the sodium salts of HCO_3^{2-} and CO_3^{2-} had deleterious effects on algal growth; such an effect in our study cannot be ruled out.

Effect of growth rate on carbon cell quota. The invariance in Q'_c with changing μ observed, representing ~45 to 50% carbon in the biomass (Fig. 2A and 3A), was identical to previous results (19, 36) and conclusively demonstrated the inapplicability of equation 1 for describing the relationship between μ and internal carbon levels when inorganic carbon is limiting and Q is defined on a dry weight or total biomass basis. Droop (9) pointed out that his original formulation of equation 1 was based on the consideration that total biomass was the proper unit for calculating Q and that only when cell volume was invariant with changing μ was it acceptable to replace biomass with cell number in this term. Yet, the general convention in most phytoplankton studies, both experimental (42) and theoretical (6), has been to use the concentration of internal nutrient per cell number as a measure of Q. The choice of biomass units actually need not be well defined because equation 1 is empirical and has no fundamental theoretical basis.

On a cellular basis there was significant variation in Q_c for both species. The degree of variation in Q_c, as indicated by the ratio of k_Q to Q_{cM} (Table 3), is somewhat more pronounced for *C. vulgaris* (ratio of k_Q to Q_{cM}, 0.16) than for *S. obliquus* (ratio of k_Q to Q_{cM}, 0.26), although there is overlap in the two values at the 95% confidence limits (Table 3). *C. vulgaris*, which is considerably smaller than *S. obliquus*, must be capable of larger relative increases in cell size with increasing μ than *S. obliquus*, assuming that cell size in the chlorophytes tested is directly proportional to Q_c. This latter inference seems reasonable for chlorophytes, which do not contain vacuoles (30, 43, 50), even though cell size was not measured in this study. Thus, cell size may be as important a parameter in dictating the potential range of Q for a particular limiting nutrient as it is in influencing the absolute value of k_Q (43).

Thus, the utility of equation 1 for describing inorganic carbon limitation in algae is restricted, and $\hat{\mu}$ and Q_{cM} must be determined experimentally, even when Q is defined as cellular carbon.

Cellular chemical ratios. The tight coupling between carbon assimilation on the one hand and nitrogen uptake (Fig. 4) and chlorophyll synthesis (Fig. 5 and 6) on the other is best represented by the lack of variance in the C-N and C-chlorophyll ratios with changing μ. The value of the ratio of C to N for both species (5 to 6) represents the lower limit possible with microbes and indicates a cell population in a well-balanced nutritional state (i.e., ~50% protein in total biomass) (Goldman, in press). Similarly, ratios of C to chlorophyll of 50 to 100 are indicative of well-nourished cells (Goldman, in press). Holm-Hansen (24) recently demonstrated that

acetone extraction (as used in this study) leads to slightly lower chlorophyll recoveries than when methanol is used, particularly for analyses on chlorophytes. Hence, in retrospect our chlorophyll values may represent a systematic underestimate; however, this would not change the trends of the data in Fig. 5 and 6 nor the general conclusion regarding the nutritional states of the two chlorophytes.

The effect of inorganic carbon limitation on cellular chemical composition is quite different than the effect of nitrogen or phosphorus limitation. Under nitrogen limitation, the nitrogen cell quota increases with increasing μ, but generally the carbon and phosphorus cellular contents either remain constant (35, 40, 41) or increase in a threshold fashion only close to μ (20). For phosphorus limitation both carbon and nitrogen cellular contents typically are independent of μ (14, 35, 40). In contrast, the cellular chlorophyll content seems to increase with increasing μ regardless of which nutrient is limiting (42).

It appears that the major effect of inorganic carbon limitation on cell physiology is not so much an effect on the chemical structure of the cells, but rather the influence of this limitation on cell size; decreases in macromolecular components and corresponding decreases in cell size are related to decreasing μ, which in turn represents an increasing degree of inorganic carbon limitation.

Algal productivity. An important consequence of the very low K_s values established for inorganic carbon-limited growth is that the steady-state level of algal carbon virtually is equal to C_{T_0} at all growth rates until just before $\hat{\mu}$ because $C_{T_0} \gg C_{T_1}$ (Fig. 1). Then algal productivity increases linearly with increasing μ and is maximum just before $\hat{\mu}$; this is followed by a rapid decrease in productivity to a value of zero at $\hat{\mu}$ (Fig. 7). Under these conditions peak productivity is concomitant with high μ. However, this situation is true only when HCO_3^- is the source of the limiting nutrient and is supplied to the culture as part of the influent liquid medium. When bubbled CO_2 which is supplied independent of the medium is the source of inorganic carbon, a decrease in algal biomass occurs with increasing μ, and peak productivity occurs when μ is considerably less than $\hat{\mu}$ (36). In attempts to optimize productivity in algal mass cultures, consideration must be given to these bioengineering constraints (15).

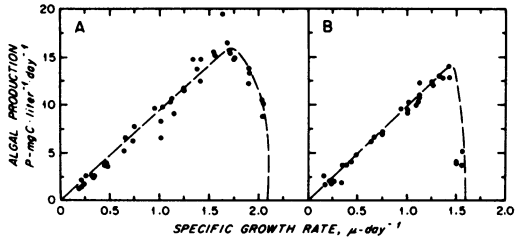

FIG. 7. *Relationship between μ and algal productivity in inorganic carbon-limited continuous cultures. (A) C. vulgaris, (B) S. obliquus. Lines were drawn by eye and are meant to demonstrate trends only.*

Conclusions. In this study the relationship between growth rate and inorganic carbon limitation was reasonably well described by the Monod equation. The Droop equation was inapplicable when total biomass was used in place of cell number in defining Q and of restricted usefulness when μ was related to cellular carbon. Both of these equations are empirical and must be used with caution in descriptions of algal growth response to nutrient limitation. Rather interestingly, the applicability of the Monod equation increases (i.e., measurable K_s values), whereas the utility of the Droop equation decreases (i.e., large ratios of k_Q to Q_M) when the limiting nutrient comprises a larger fraction of cellular biomass. Phosphorus, which constitutes a minute fraction of cellular biomass, and carbon, which makes up ~50% of the total cellular biomass, represent the extreme examples of this concept.

The affinity that algae have for inorganic carbon is high enough to prevent distinguishing between CO_2 uptake and HCO_3^- uptake on the basis of chemical equilibrium considerations. The major factors controlling inorganic carbon uptake are physical mass transpsort bottlenecks at gas-liquid interfaces and the photosynthetic process itself. The mass flux of CO_2 or HCO_3^- or both across cell membranes does not appear to be a rate-limiting step.

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