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Measurement of Carbon Dioxide Compensation Points of Freshwater Algae[1]

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ABSTRACT

A technique is described for the measurement of total dissolved inorganic carbon by acid release as CO_2 followed by its conversion to methane and detection by flame ionization in a modified gas chromatograph. This method was used to determine the dissolved inorganic carbon concentration reached at compensation point when algae were allowed to photosynthesize in a closed system in a buffer at known pH, and the CO_2 compensation point was calculated from this concentration. The CO_2 compensation points of 16 freshwater algae were measured at acid and alkaline pH in air-saturated medium: at acid pH the CO_2 compensation points ranged from 4.8 to 41.5 microliters per liter while at alkaline pH they ranged from 0.2 to 7.2 microliters per liter. Removal of O_2 from the medium caused a slight lowering of compensation point at acid pH but had little effect at alkaline pH. These low, O_2-insensitive compensation points are characteristic of C_4 plants. It is suggested that these low CO_2 compensation points are maintained by an active bicarbonate uptake by algae especially at alkaline pH.

The CO_2 compensation point of a green plant is the CO_2 concentration at which the rate of photosynthetic CO_2 uptake is equal to the rate of respiratory CO_2 loss. It has been determined in higher plants by measurement of the CO_2 concentration in a closed system by using the IRGA.[2] The compensation points of some algae have been measured in a similar fashion: by IRGA determination of the CO_2 concentration in air either circulated through algal suspensions (8, 27) or passed over thin layers of algae suspended on wet membrane filters (12). Since this system only measures CO_2 in the gas phase and rapid equilibration of CO_2 between the gas and aqueous phases only occurs between pH 4 and 5, algal compensation points have been measured in this pH range. However, many algae grow and photosynthesize in alkaline media and it would be useful to measure compensation points of algae at their photosynthetic pH optimum. Measurement of the CO_2 equilibrium between algal cells and the medium at an alkaline pH is difficult because the concentration of free CO_2 is very low, often below the limits of detection of the IRGA (25).

In light of these possible limitations of the IRGA method we have used an alternative technique to measure the CO_2 compensation points of a number of freshwater algae. A sensitive gas chromatographic technique using a flame ionization detector was adapted for measuring low levels of dissolved inorganic carbon DIC and the equilibrium DIC reached by allowing algae to photosynthesize in a closed system, in a buffer at a known pH, was measured. The proportion of free CO_2 in buffer at this equilibrium concentration, *i.e.* the CO_2 compensation point, was calculated using the equation of Buch (4).

MATERIALS AND METHODS

Axenic cultures of *Anabaena flos-aquae* (Lyngbye) Breb. (1444), *Anacystis nidulans* Richt. (1550), *Coccochloris peniocystis* Kutz. (1548), *Chlorella pyrenoidosa* Chick (395), *Chlorella vulgaris* Beijerinck (259), *Chlamydomonas reinhardtii* Dangeard (90), *Cladophora glomerata* (L.) Kutz (1486), *Navicula minima* var. *atomoides* (Grun.) (391), and *Navicula pelliculosa* (Bréb.) Hilse (668) were obtained from the culture collection of algae at the University of Texas, Austin (culture collection numbers in parentheses). *Phormidium molle* was a gift from Dr. S. R. Brown, Queen's University, Kingston, Ontario, Canada. Unialgal cultures of *Mougeotia* sp. (15-2360), *Stigeoclonium* sp. (15-2600), and *Zygnema* sp. (15-2695) were obtained from Carolina Biological Supply Co., Burlington, N.C. (catalog numbers in parentheses). *Chlorella fusca* (211/8p) was obtained in axenic culture from the Culture Centre of Algae and Protozoa, Cambridge, U.K. Axenic cultures of *Asterionella formosa* (Clone Fra Af) and *Cyclotella meneghiniana* (Clone Cy Oc2) (22) were obtained from Dr. D. G. Wallen, University of Windsor, Ontario, Canada.

All of the blue-green algae were grown as previously described (6); diatoms were grown on modified Tris-buffered freshwater medium (2); and unicellular green algae were grown on the freshwater medium or as previously described (5). Filamentous green algae were grown on either the medium of Smith and Wiedeman (17) or Bristol's medium (19) modified as follows: KH_2PO_4 was omitted, $Na_2SiO_3 \cdot 9H_2O$ was added to give a final concentration of 40 mg/l, and the medium was buffered with 600 mg/l Tris-HCl (pH 7.8) or 500 mg/l Hepes-NaOH (pH 7.8); the trace element solution of the freshwater medium was used.

The unicellular green algae and *Navicula pelliculosa* were grown in 250-ml Erlenmeyer flasks containing 100 ml of growth medium on a shaker. *Asterionella formosa*, *Cyclotella meneghiniana*, and the filamentous green algae were grown in 2.8-liter Fernback flasks containing 1 liter of growth medium with periodic shaking by hand. The blue-green algae were grown at room temperature (26–29 C) and illuminated continuously with cool-white fluorescent lamps. The light intensity was 2.5 to 3.5 mw/cm^2 at culture flask level. The green algae and diatoms were grown at 20 C using the same light intensity with a photoperiod of 16 h light/8 h dark.

Unicellular algae were harvested during the linear growth phase and concentrated by centrifugation at 1,000 to 8,000*g* for 10 min. Filamentous green algae were harvested on cotton gauze. The cells were resuspended in 50 mM buffer (Mes-NaOH or KH_2PO_4 for pH 5.5–pH 5.9 and K_2HPO_4, Hepes-NaOH or Tris-HCl for pH 7.5–pH 8.1) which had been previously flushed with "CO_2-free" N_2 or air to establish low or air-saturated levels of dissolved O_2. CO_2-free gases were obtained by passage over KOH or NaOH

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[2] Abbreviations: IRGA: infrared gas analyzer; DIC: dissolved inorganic carbon; FID: flame ionization detector.

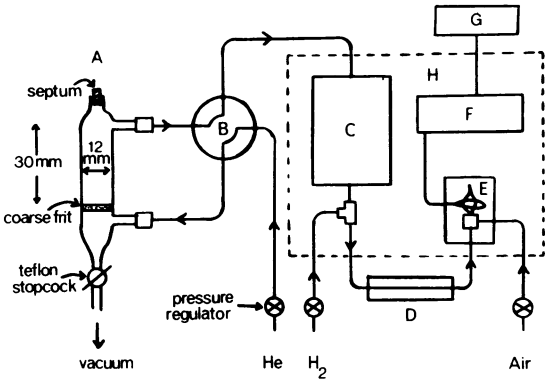

FIG. 1. Schematic diagram showing modifications to the gas chromatograph for measuring dissolved CO_2 as methane. A: gas-stripping column; B: four-way valve; C: oven containing a 6-mm o.d. × 1.4-m coiled glass column; D: oven containing nickel catalyst in a 6-mm o.d. × 10-cm stainless steel tube; E: FID; F: electrometer; G: Varian A-25 recorder (2 mv full scale, 63.5 cm/h); H: Varian Aerograph 2740 gas chromatograph.

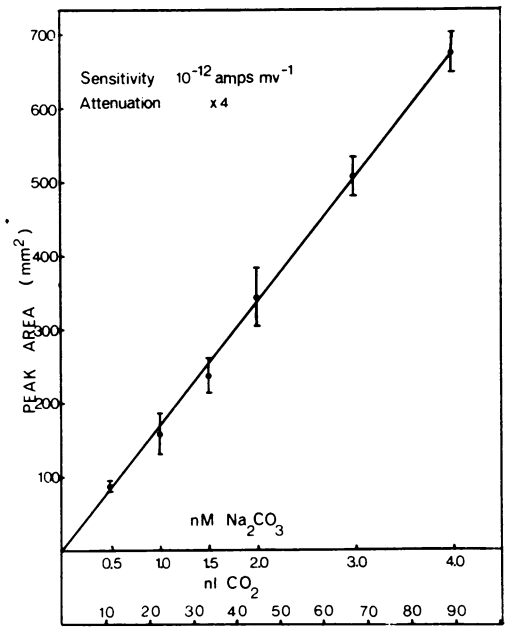

FIG. 2. Recorder response (peak area) *versus* volume (μl) of 1 mM Na_2CO_3 injected.

pellets. Cells were drawn up into 5-ml gas-tight Hamilton syringes, sealed, and incubated with shaking in an illuminated water bath (4.0–4.5 mw/cm^2). Diatoms and green algae were incubated at 20 C and blue-green algae at 28 C for CO_2 compensation point determinations. At 10- to 15-min intervals the total DIC of the cell-free medium was determined by passing the cell suspension through an AP20 or AP25 prefilter and a 0.8 μM filter held in a 13-mm-diameter Swinnex membrane filter holder (Millipore Corp.) attached to the syringe. The cell-free medium was injected directly into the gas-stripping column of the gas chromatograph through a hypodermic needle to minimize contamination with atmospheric CO_2.

The flow scheme of the modified gas chromatograph used in this study is shown in Figure 1. The cell-free sample was mixed in the gas-stripping column with ~0.2 ml 50% H_3PO_3, and the dissolved gases were stripped from solution with helium carrier gas (20) onto a coiled glass column (1.4 m × 6.0 mm o.d.) packed with Porapak Q (Dow Chem. Co.) maintained at 60 C. The gas chromatograph was operated under the following conditions: helium carrier gas flow, 25 ml/min; H_2 gas flow, 25 ml/min; air flow, 275 ml/min; injection port temperature, 110 C; and detector temperature, 125 C. The gas emerging from the column was mixed with H_2 and passed over a nickel catalyst maintained at 350 to 400 C (28). The response of the gas chromatograph to CO_2 was calibrated by injecting μl amounts of Na_2CO_3 solutions prepared with boiled distilled H_2O.

Photosynthetic rates of cell suspensions used in compensation point determinations were measured as O_2 evolution using a thermostatted Clark-type O_2 electrode (Hansatech Ltd., Kings Lynn, Norfolk, U.K.) calibrated as previously described (7). Chl content of algal suspensions was determined after extraction with methanol (10) or 90% acetone (11).

RESULTS

An illustration of sensitivity and accuracy of the gas chromatographic technique developed in this study is given by the standard curve of peak area against DIC concentration (Fig. 2) which was obtained by injecting μl amounts of 1 mM Na_2CO_3. The standard deviation at the 2-nmol Na_2CO_3 level was 8.5% (N = 31). Generally, 1 ml of cell-free medium was injected onto the gas chromatograph so that peak area could be read off directly as nl CO_2/ml or μl CO_2/l. This CO_2-methanation technique is extremely sensitive and has a detection limit of ~5 nl CO_2. It is at least 3 orders of magnitude more sensitive than other techniques based on acid release of total CO_2 in aqueous samples using either thermal conductivity (18) or IRGA detectors (9).

Algae contained in a closed system in the light and suspended in phosphate buffer at pH 7.9 caused an initial rapid depletion of the DIC of the medium. This depletion could be due to an uptake of HCO_3^- or an uptake of free CO_2 provided by the dehydration of HCO_3^-. In the latter case the uptake of carbon would be limited by the rate of spontaneous dehydration of HCO_3^- at this pH. There are two published values of the rate constant of this reaction (14, 15) but empirical measurements of the dehydration rate were made under our particular conditions of pH, temperature, and buffer concentration. The spontaneous bicarbonate dehydration rate was determined at 25 C by sparging 50 mM phosphate buffer (pH 7.9) solution with CO_2-free N_2 at 950 or 1900 ml/min and measuring the decrease of DIC with time (Fig. 3). The rate of loss of CO_2 was unaffected by the rate of sparging at these sparging

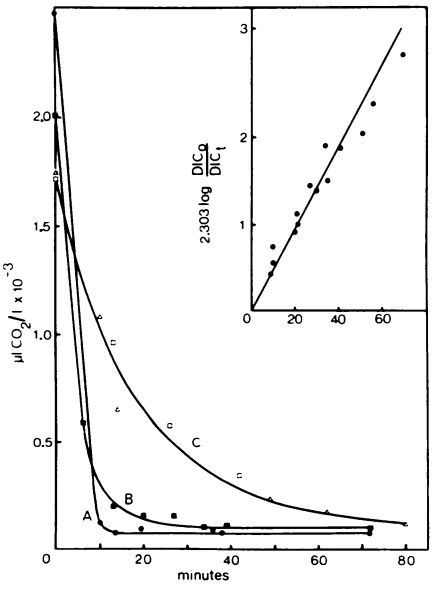

FIG. 3. DIC (μl CO_2/l) depletion kinetics of (A) *Phormidium*, (B) *Chlorella vulgaris*, and (C) 50 mM phosphate buffer (pH 7.9) gassed with CO_2-free N_2 at 950 ml/min and 1,900 ml/min. Inset: plot of 2.303 log DIC_o/DIC_c *versus* time (min).

Table I. *Compensation Points of Several Species of Freshwater Algae in Acid Media at Two O_2 Concentrations*

Algae	pH	Oxygen Concentration			
		2%		21%	
		Equilibrium DIC	Compensation point	Equilibrium DIC	Compensation point
		μl/l			
Chlorella pyrenoidosa	5.6	7.0	5.9	6.8	5.8
C. vulgaris	5.6	8.4	7.1	5.6	4.8
C. fusca	5.4	11.5	10.4	23.4	21.1
Chlamydomonas reinhardtii	5.5	35.0	30.7	25.9	22.7
Cladophora glomerata	5.6	18.3	15.5	32.0	27.1
Mougeotia sp.	5.5	28.0	24.6	47.3	41.5
Stigeoclonium sp.	5.8	30.0	23.4	36.8	28.8
Zygnema sp.	5.5	19.3	16.9	29.6	26.0
Asterionella formosa	5.3	15.0	13.8	21.3	19.5
Cyclotella meneghiniana	5.5	25.9	22.7	19.4	17.0
Navicula minima	5.5			33.6	29.5
N. pelliculosa	5.7	17.4	14.3	25.6	21.0
Anacystis nidulans	5.4	22.0	20.0	36.6	33.3
Anabaena flos-aquae	5.5	22.5	19.7	25.3	22.2
Coccochloris peniocystis	5.7	20.1	16.5	18.0	14.8
Phormidium molle	5.6	17.1	14.5	17.8	15.1

rates and the pH of the solution remained constant throughout the course of the experiments. A plot of rate of DIC loss with time (Fig. 3 inset) illustrates the pseudo-first order nature of the reaction. The dehydration rate constant under these conditions was calculated to be 0.8×10^{-3}/s, which is in close agreement with published values of 0.9×10^{-3}/s (14) and 0.7×10^{-3}/s (15).

The measured initial rate of DIC depletion caused by photosynthesizing algae in a closed system is somewhat greater than would be expected if the algae took up free CO_2 at this pH. In the experiment shown in Figure 3, *Phormidium* caused DIC depletion of the medium at an initial rate of 260 μl CO_2/l·min, while *Chlorella* caused depletion at the rate of 236 μl CO_2/l·min. Using the experimentally determined value of the bicarbonate spontaneous dehydration rate constant, the calculated over-all rate of supply of CO_2 over the concentration range 2,000 μl/l to 500 μl/l was 52 μl/l·min. This experiment clearly demonstrates that *Chlorella* and *Phormidium* can remove DIC from an alkaline medium 4.5- to 5-fold faster than CO_2 can be supplied to the algae by spontaneous dehydration of HCO_3^- under these conditions. They appear, therefore, to be using HCO_3^- ions directly.

Suspensions of each of 16 species of algae were sealed in glass syringes in media with an initial concentration of 200 to 600 μl/l DIC and allowed to photoassimilate DIC. The DIC concentration was determined at timed intervals until equilibrium was reached and maintained for 30 min or more. The gas-tight syringes used in these experiments allowed CO_2 to leak in at rates of 10 to 30 μl/l·h but this did not affect the establishment of an equilibrium with DIC or raise the DIC concentration at equilibrium. The photosynthetic rates of the algal suspensions were determined, at saturating bicarbonate concentration and light intensity, prior to each experiment and were in the range 40 to 250 μmol O_2/mg Chl·h. The concentration of DIC at equilibrium was measured for each alga at acid and alkaline pH in air-saturated medium and at low O_2 concentration.

The DIC concentration at equilibrium varied between algae and also varied with the pH of the medium. At acid pH in air-saturated medium the equilibrium DIC ranged from 5.6 μl/l for *Chlorella vulgaris* to 47.3 μl/l for *Mougeotia* (Table I) whereas at alkaline pH these concentrations were considerably higher, ranging from 7.7 μl/l for *Phormidium* to 168 μl/l for *Navicula minima* (Table II). These DIC concentrations are the sum of the concentrations of HCO_3^- and CO_2 expressed as CO_2. The concentration of free CO_2 is a fraction of this total and varies with the pH of the medium. The free CO_2 concentration at the DIC equilibrium was therefore calculated using the equations of Buch (4).

The compensation points at acid pH ranged from 4.8 to 41.5 μl/l. The lowest values recorded were those for *Chlorella pyrenoidosa* and *C. vulgaris* of 5.8 and 4.8 μl/l, respectively, whereas those of most of the other algae lay in the range 15 to 20 μl/l (Table I). A lowering of the O_2 concentration of the medium at this pH did not have marked effects on the compensation points. Those of the unicellular green algae, *Chlorella* species and *Chlamydomonas*, the blue-green algae *Anabaena*, *Coccochloris*, and *Phormidium*, and the diatom *Cyclotella* were apparently unaffected by changes in O_2 concentration whereas the compensation points of other species decreased at most by 50% (Table I).

In contrast to the compensation points at acid pH, the CO_2 compensation points at alkaline pH were uniformly low (Table II). At air-saturated concentrations of O_2 the compensation points of all species lay in the range 0.2 to 7.2 μl/l and a lowering of the O_2 concentration had no marked effects on any of the compensation points (Table II).

DISCUSSION

The compensation points obtained in this study for freshwater algae at acid pH are similar to values obtained for some of the same species by use of the IRGA. The compensation points of *Chlorella vulgaris* and *C. pyrenoidosa* for example were found to be 4.8 and 5.7 μl/l, respectively, whereas other workers have reported values of 10 μl/l or less (8, 27). Similarly, the compensation points of the filamentous green algae *Cladophora*, *Mougeotia*, *Stigeoclonium*, and *Zygnema* at acid pH range from 26.0 to 41.0 μl/l while that reported for *Nitella* at acid pH is 32 μl/l (3).

These results also demonstrate that compensation points of algae decrease with an increase in pH (*cf* Tables I and II). The uniformly low compensation points at alkaline pH are similar to those obtained by Lloyd *et al.* (12) by use of the IRGA with thin films of algae suspended in small volumes of medium presumably at an alkaline pH. However, some of the compensation points measured in this study (Table II) are very low and would be at the extreme lower limit of detection of the IRGA.

Table II. *Compensation Points of Several Species of Freshwater Algae in Alkaline Media at Two O_2 Concentrations*

Algae	pH	Oxygen Concentration			
		2%		21%	
		Equilibrium DIC	Compensation point	Equilibrium DIC	Compensation point
		μl/l			
Chlorella pyrenoidosa	7.9	11.1	0.3	25.4	0.7
C. vulgaris	7.7	22.3	0.9	30.0	1.3
C. fusca	7.7	19.5	0.8	43.8	1.9
Chlamydomonas reinhardtii	8.1	17.8	0.3	39.8	0.7
Cladophora glomerata	7.7	63.5	2.7	94.5	4.1
Mougeotia sp.	7.7	75.6	3.2	103.3	4.4
Stigeoclonium sp.	7.8	38.0	1.3	46.0	1.6
Zygnema sp.	7.8	44.6	1.5	85.5	2.9
Asterionella formosa	7.7			105.0	4.5
Cyclotella meneghiniana	7.6	25.0	1.3	28.5	1.5
Navicula minima	7.7			168.0	7.2
N. pelliculosa	7.8	10.4	0.4	36.9	1.3
Anacystis nidulans	8.0	9.6	0.2	9.5	0.2
Anabaena flos-aquae	7.6	17.3	0.9	19.2	1.0
Coccochloris peniocystis	7.7	20.8	0.9	17.0	0.7
Phormidium molle	7.8	7.5	0.3	7.7	0.3

No marked effects on the compensation points of the algae were detected on changing the O_2 concentration of the medium. Some effect of O_2 on compensation point of filamentous green algae was observed at acid pH (Table I), but at alkaline pH there is little difference between the compensation points of the algae under N_2 and those under air-saturated levels of O_2. The algae with the least sensitivity to O_2 concentration appeared to be the unicellular green algae and the blue-green algae (Tables I and II).

The insensitivity of the compensation points of algae to O_2 in the concentration range 2 to 21% has been reported previously (12). These observations are greatly outweighed by a large number of early studies which demonstrate an inhibition of photosynthesis in algae by O_2, that is a Warburg effect (26). These inconsistencies may only be apparent, however, since it has been pointed out by Turner and Brittain (24) that early demonstrations of the Warburg effect in algae generally resulted from the use of high concentrations of O_2 (21–100%) and the use of algae grown on high O_2 concentrations, a procedure which is known to suppress photosynthesis markedly at low CO_2 concentrations (24).

These low, O_2-insensitive compensation points of algae are characteristic of C_4 plants, and are consistent with previous findings that algae release less CO_2 in the light than in the dark (5). Only the blue-green algae have been reported to have any capability of fixing CO_2 by the C_4 pathway (6) and most algae are considered to be C_3 plants (23). It is evident from our results and those of others (21) that algae are capable of maintaining photosynthesis at alkaline pH in media of low CO_2 concentration. To maintain this equilibrium the algae may take up HCO_3^- as HCO_3^- is the predominant inorganic carbon species at this pH (16). An active uptake of HCO_3^- has been elegantly demonstrated by Lucas (13) in the filamentous alga *Chara*, and Badger *et al.* (1) have provided evidence for HCO_3^- transport in *Chlamydomonas* and *Anabaena*. There is also some evidence in the present results (Fig. 3) that the rate of carbon uptake at alkaline pH by other algae can be satisfactorily explained only by HCO_3^- uptake. The active uptake of HCO_3^- ions into the algal cell would serve as a CO_2-concentrating mechanism to maintain photosynthesis under conditions where the external CO_2 concentration is too low to allow any direct uptake of CO_2 into the cell. The over-all effect of an active HCO_3^- transport system would be to give the alga the physiological characteristics of a C_4 plant while the initial carboxylation enzyme system is that of a C_3 plant.

The compensation point is a measure of the equilibrium between the influx of inorganic carbon and the efflux of CO_2 by the cell and may not be affected by the total flux of the various forms of inorganic carbon across the cell membrane. These data which indicate that algae have low compensation points cannot therefore be interpreted to support the view that no photorespiration occurs in algae (12).

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