Salicylhydroxamic Acid (SHAM) Inhibition of the Dissolved Inorganic Carbon Concentrating Process in Unicellular Green Algae

Arun Goyal* and N. Edward Tolbert
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

ABSTRACT
Rates of photosynthetic O2 evolution, for measuring $K_{eq}(CO_2 + HCO_3^-)$ at pH 7, upon addition of 50 micromolar HCO_3^- to air-adapted Chlamydomonas, Dunaliella, or Scenedesmus cells, were inhibited up to 90% by the addition of 1.5 to 4.0 millimolar salicylhydroxamic acid (SHAM) to the aqueous medium. The apparent $K_{eq}$(SHAM) for Chlamydomonas cells was about 2.5 millimolar, but due to low solubility in water effective concentrations would be lower. Salicylhydroxamic acid did not inhibit oxygen evolution or accumulation of bicarbonate by Scenedesmus cells between pH 8 to 11 or by isolated intact chloroplasts from Dunaliella. Thus, salicylhydroxamic acid appears to inhibit CO2 uptake, whereas previous results indicate that vanadate inhibits bicarbonate uptake. These conclusions were confirmed by three test procedures with three air-adapted algae at pH 7. Salicylhydroxamic acid inhibited the cellular accumulation of dissolved inorganic carbon, the rate of photosynthetic O2 evolution dependent on low levels of dissolved inorganic carbon (50 micromolar NaHCO_3), and the rate of CO2 fixation with 100 micromolar [14C] HCO_3-. Salicylhydroxamic acid inhibition of O2 evolution and CO2 fixation was reversed by higher levels of NaHCO_3. Thus, salicylhydroxamic acid inhibition was apparently not affecting steps of photosynthesis other than CO2 accumulation. Although salicylhydroxamic acid is an inhibitor of alternative respiration in algae, it is not known whether the two processes are related.

Unicellular green algae, Chlamydomonas (4, 21), Chlorella (18), Dunaliella (2, 14), Scenedesmus (11, 23, 28), and cyanobacteria such as Anabaena (29) and Synechococcus (3, 24), when grown with or adapted to low air levels of CO2 in the light, exhibit a $K_{eq}$(DIC)$^2$ for photosynthetic O2 evolution with low levels of external DIC by accumulating inorganic carbon inside the cells. This process has been termed a 'dissolved inorganic carbon concentrating mechanism' or 'DIC pump.' When microalgae are grown with elevated levels of CO2 (air supplemented with 1 to 5% CO2), the light-dependent intracellular accumulation of inorganic carbon is suppressed. During DIC accumulation, either or both CO2 or HCO_3-

1 Supported by McKnight Foundation Photosynthesis training grant and the Michigan Agricultural Experiment Station.
2 Abbreviations: $K_{eq}$(DIC), CO2 plus HCO_3- concentration at a given pH at which the rate of DIC dependent photosynthetic O2 evolution is half-maximal; DIC, dissolved inorganic carbon; CA, carbonic anhydrase; PCV, packed cell volume; SHAM, salicylhydroxamic acid.

MATERIALS AND METHODS

Organisms and Growth Conditions
Chlamydomonas reinhardtii 137 cells were grown photoautotrophically in minimal media at 26 ± 2°C with continuous shaking and bubbling with 5% CO2 in air (27). Dunaliella tertiolecta (CSIRO Marine Laboratories, Hobart, Tasmania, Australia) was maintained and cultured on a defined medium (15) with 0.17 m NaCl as described previously (12, 14). The photon flux density was 150 μE m^-2 s^-1 with a light/dark regime of 16/8 h. The pH of the algal growth medium started around 7 and drifted lower to near 5 during growth of the cultures. While in the exponential growth phase and 1 d before use, cultures were diluted with fresh growth media and aerated only by shaking for 3 to 18 h to develop DIC pumps. Cultures were harvested by centrifugation (1000g x 10 min); the cell pellet was washed once with the fresh growth medium (pH 6.8), and centrifugation was repeated. Finally, the cell pellet was resuspended in a small volume of assay buffer (50
mm Hepes at pH 7.0 or 7.2), kept on ice, and used within 2 h. Chl was estimated on an aliquot after ethanol extraction. *Scenedesmus obliquus* from N. I. Bishop (Dept. of Botany, Oregon State University, Corvallis, OR) were maintained on slants prepared with *Scenedesmus* medium (6) containing 0.5% glucose, 0.25% yeast extract, and 2% agar (NYG) (5). Algae were grown photoautotrophically in *Scenedesmus* medium (6) in thermostated cylinders (800 mL) at 32°C with continuous bubbling with 5% CO₂ in air. The cylinders were illuminated continuously from both sides by two banks of fluorescent lights that provided the algae an intensity of 100 μE·m⁻²·s⁻¹. The following procedure was used to activate the DIC pumps in *Scenedesmus* where exogenous DIC, particularly at alkaline pH, would slow down the rate of activation. Cultures were harvested by centrifugation (1000g × 10 min) in midexponential phase of growth, the cell pellet was washed once with water, and centrifugation was repeated. The final cell pellet was resuspended to a volume of 5 μL PCV mL⁻¹ in 20 mm phosphate buffer at pH 7.2 or dibasic sodium phosphate at pH 9.0. Five μL PCV was equal to 25 μg equivalent of Chl. Cells were shaken without aeration for 3 to 4 h in light at either pH 7.2 or 9.0 to develop the DIC pumps. The phosphate buffer at pH 7.2 prevented a rise in pH from alkalization of the medium during DIC uptake. When starting at pH 9.0 with Na₂HPO₄, the pH did not increase to over 10. Before tests, an aliquot was centrifuged briefly in a microfuge, and the cells were resuspended in the same volume of fresh phosphate at the indicated pH of 7.2 or 9.0. Hepes buffers were prepared fresh daily in boiled glass distilled water, and the pH was adjusted with crystals of AR grade KOH in an effort to keep the DIC to a minimum (28). The phosphate buffer at pH 7.2 was prepared by dissolving monobasic sodium phosphate in boiled water and adjusting the pH with dibasic sodium phosphate. All solutions were continuously sparged with N₂ until used.

Intact chloroplasts from *Dunaliella* were isolated as described in reference (12) and inorganic carbon uptake measured as in (14, 20).

**Photosynthetic Oxygen Evolution**

Photosynthetic CO₂-dependent oxygen evolution was measured with a Clark-type oxygen electrode (Rank Brothers, Cambridge, UK). Harvested cells were diluted from a concentrated suspension to 25 μg Chl·mL⁻¹ in 50 mm Hepes or 20 mm phosphate buffered at pH 7.0 or 7.5 for *Chlamydomonas* or *Dunaliella*. *Scenedesmus* cells were assayed in 20 mm phosphate buffer at pH 7.2 or 20 mm Na₂HPO₄ at pH 9.0.

Cell suspensions were then illuminated with 800 μE·m⁻²·s⁻¹ light at 25°C in a thermostated O₂ electrode chamber until the endogenous DIC was consumed by the cells, as judged by the cessation of O₂ evolution (Fig. 1, ref. 28). Rates of CO₂-dependent O₂ evolution were then measured after addition of known amounts of NaHCO₃. As an indication of DIC pump activity, the rate of photosynthetic O₂ evolution from the addition of DIC was used to calculate KD₅₀(DIC) as described previously (21). Where indicated, SHAM was added to the reaction mixture from a stock solution of 0.45 mM in ethanol. Because SHAM has low solubility in water, the effective concentration in solution would be lower than the amount added. The dilution factor for the SHAM stock was at least 100-fold and the carry over of 1% or less ethanol did not effect the rate of O₂ evolution. Nevertheless, 1% ethanol was added to controls without for comparative purposes. All experiments were run at least in duplicate, and all results for each alga were confirmed with cultures grown at three different periods.

**14CO₂ Fixation**

The 14CO₂ fixation was measured in a 2 mL cell suspension in 25 mm Hepes-KOH buffer (pH 7.5) containing cells equivalent to 40 μg Chl. The algal suspension was continuously stirred in a thermostated cuvette (25°C) and illuminated with 800 μE·m⁻²·s⁻¹ from a Kodak projector. The reaction was initiated by adding an aliquot from a stock solution of H⁺¹⁴CO₃⁻ (0.5 mCi/mmole, Research Products International, USA) to provide 2 mm and every 5 min an additional aliquot was added to give 1 mm bicarbonate. Aliquots of 0.45 m SHAM in ethanol was added to the cell suspension 1 min prior to addition of H⁺¹⁴CO₃⁻. To the control algal suspension, an equal amount of ethanol was added. After 5, 10, or 15 min, the fixation was terminated by adding 1 mL hot aqueous methanol 80% (v/v). Radioactivity was determined in 50 μL aliquot after releasing unfixed inorganic carbon by adding 0.45 mM acetic acid (0.5 N) and warming the vials at 80°C for 2 h.

**Inorganic Carbon Uptake**

DIC uptake by algal cells was estimated by silicone oil filtration (4, 14, 20). Assays were performed in light of 400 μE·m⁻²·s⁻¹ at 25°C in 400 μL microfuge tubes in a Beckman Microfuge II. Cell suspension was placed in a microfuge tube over a gradient of 25 μL of 1 M glycine and 0.75% (w/v) SDS at the bottom, overlaid with 75 μL of a silicone oil mixture (four parts of Wacker AR-200 to one part of Wacker AR-20 silicone oil from SWS Silicones, Adrian, MI). A large evaporating dish filled with cold water was placed on the top of the open microfuge with the loaded tubes so that heat from the illumination did not warm the cell suspension or change the viscosity of the silicone oil. After preillumination for 1 min, DIC uptake was initiated by the addition of 5 μL of NaH¹⁴CO₃ (Research Products International; specific radioactivity 45 mCi·mmole⁻¹ for *Chlamydomonas*, and 35 mCi·mmole⁻¹ for *Scenedesmus*), and the incubation was terminated after 30 to 60 s by turning on the microfuge. Where appropriate, 1.5, 2, or 4 mM SHAM was added to the cell suspension at the beginning of the 1 min preillumination. The DIC uptake was calculated by estimating the cell volume using ¹⁴C-sorbitol and ³H₂O as previously described (21). The final concentration of NaHCO₃ in the medium was about 150 μM. Inorganic carbon accumulation was calculated by subtracting the acid stable radioactivity from the total ¹⁴C uptake.

**RESULTS**

**Effect of SHAM on Photosynthetic Oxygen Evolution**

After cultures grown on high CO₂ were adapted in air for 24 h, cells of both *Chlamydomonas* and *Dunaliella* at pH 7.0...
had a well developed DIC pump for a $K_{0.5}(\text{DIC})$ with 1 to 10
$\mu\text{M}$ DIC. When these algae were supplied with 50 to 75 $\mu\text{M}$
$\text{HCO}_3^-$ at pH 7.0 or 7.5, oxygen was evolved at a maximal
rate of about 90 to 98 $\mu\text{mol} \text{O}_2 \cdot \text{mg}^{-1} \text{Chl} \cdot \text{h}^{-1}$ (Table I; Fig.
1), until the DIC concentration was depleted to about 5 to 10
$\mu\text{M}$. When 3 $\text{mM}$ SHAM was added to the culture along with
75 $\mu\text{M}$ $\text{HCO}_3^-$, the rate of photosynthetic oxygen evolution
was inhibited about 70% for *Chlamydomonas* and 60% for
*Dunaliella*, but oxygen evolution would continue at this re-
duced rate for a longer period until most of the DIC was used
up and DIC concentration became rate limiting. The addition
of an excess amount of $\text{HCO}_3^-$ (0.5–2 $\text{mM}$) at the third arrow
in Figures 1 and 2 reversed the SHAM inhibition of photo-
synthetic oxygen evolution (Table I). As explained later, the
apparent $K_s$(SHAM) was around 2.5 $\text{mM}$ (Fig. 3A), but higher
concentrations of SHAM were not used to prevent inhibition
of photosynthesis. This reversal of SHAM inhibition of pho-
synthetic oxygen evolution by a high level of $\text{HCO}_3^-$ sug-
gests that SHAM inhibited the DIC pumps but not photosyn-
thetic CO$_2$ reduction (Fig. 3B). With a higher concentration
of DIC at pH 7 to 7.5 enough CO$_2$ would be available to
diffuse into the cell to sustain maximum photosynthetic oxy-
gen evolution.

Since both these algae belong to the same family and appear to
have similar DIC-concentrating mechanism (2, 14), the
effect of SHAM on *Scenedesmus* adapted to air at two differ-
ent pH was also tested. *Scenedesmus* adapted at acidic pH

<table>
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<td>75 $\mu\text{M}$ $\text{HCO}_3^-$</td>
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<td><em>C. reinhardtii</em></td>
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<td>No SHAM</td>
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<td>pH 7.0</td>
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<td>97</td>
</tr>
<tr>
<td><em>D. tertiolecta</em></td>
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<td>100 $\mu\text{M}$ $\text{HCO}_3^-$</td>
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<td><em>S. obliquus</em></td>
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*With 2000 $\mu\text{M}$ NaHCO$_3^-$.

Figure 1. Photosynthetic oxygen evolution by air-adapted *C. rein-
hardtii* or *D. tertiolecta* with a DIC pump. Cells containing 50 $\mu\text{g}$ Chl
were mixed with 2 mL 50 $\text{mM}$ Hepes buffer at pH 7.0 or 7.5 in the
cuvette of an O$_2$ electrode in the light. First the cells were allowed to
deplete the endogenous DIC in the medium. A low level of 75 $\mu\text{M}$
NaHCO$_3^-$ was added (the first arrow) for 1 min to establish $V_{\text{max}}$ and
then 3 $\text{mM}$ SHAM (final concentration) and additional 75 $\mu\text{M}$ NaHCO$_3^-$
(the second arrow) was added. After measuring SHAM inhibition,
$V_{\text{max}}$ was restablished with high $\text{HCO}_3^-$ (0.5 $\text{mM}$).

Figure 2. Photosynthetic oxygen evolution by *S. obliquus* adapted
in air for 3 to 4 h in the light at either pH 7.2 or 9.0 in 20 $\text{mM}$ sodium
phosphate buffer to develop a DIC pump. Cell suspensions with
approximately 25 $\mu$g Chl per mL were shaken continuously in air and
light for 3 to 4 h to develop DIC pumps. An aliquot of 2 $\text{mL}$ of
suspension was pelleted by centrifugation for about 10 s in a micro-
fuge and the pellet was resuspended in 2 mL of phosphate buffer
pregassed with nitrogen and put in the O$_2$ electrode. First the cells
were allowed to deplete endogenous DIC, and then the rate of O$_2$
 evolution with 100 $\mu\text{M}$ $\text{HCO}_3^-$ was measured until depleted. Then the
inhibited rate was measured with 1.5 $\text{mM}$ SHAM and 100 $\mu\text{M}$ $\text{HCO}_3^-$.
$V_{\text{max}}$ was restored with excess $\text{HCO}_3^-$ (1 or 2 $\text{mM}$).
SHAM inhibition of DIC-pump by algae

Figure 3. Effect of SHAM concentration on photosynthesis in Chlamydomonas. A, Oxy- gen evolution was measured at pH 7.0 as described in "Materials and Methods." The bicarbonate concentrations used for the assay were 50 μM for air-adapted cells (○), or 250 mM for CO₂-grown cells (●). The control (as 100%) rates of O₂ evolution were 150 μmol O₂·mg⁻¹ Chl·h⁻¹ for air adapted cells and 107 μmol O₂·mg⁻¹ Chl·h⁻¹ for CO₂-grown cells. B, ¹⁴CO₂-fixation was measured by the same assay conditions at pH 7.5 except that cells with 20 μg Chl·ml⁻¹ was used. Reaction was initiated by adding 2 mM [¹⁴C]HCO₃⁻ and continued by adding 1 mM H⁺CO₂⁻ every 5 min. Aliquots of cell suspension were quenched with 1 mL 80% (v/v) hot methanol. The rate of ¹⁴CO₂ fixation in control (as 100%) without SHAM was 115 μmol ¹⁴CO₂·mg⁻¹ Chl·h⁻¹ + 2.5 mM SHAM (○), or + 5 mM SHAM (●).

This has predominantly a DIC pump for external CO₂, whereas at alkaline pH DIC uptake is predominantly by a bicarbonate pump (11, 28). SHAM (1.5 mM) inhibited about 70% of the photosynthetic oxygen evolution at pH 7.2 by Scenedesmus cells that had been adapted at pH 7.2 to low CO₂ conditions (Fig. 2). These results were similar to those with Chlamydomonas and Dunaliella. However, with Scenedesmus, which were air-adapted at pH 9 to develop a plasmalemma HCO₃⁻ transporter (28), photosynthetic O₂ evolution was only inhibited by SHAM about 28% at pH 7.2 or 14% at pH 9.0 (Table 1; Fig. 2). Inhibition of photosynthetic O₂ evolution by SHAM was reversed by 1 to 2 mM HCO₃⁻ for the cells adapted at pH 7.2 and tested at pH 7.2 or adapted at pH 9.0 and tested at pH 7.2. Cells air-adapted at pH 9.0 and tested at pH 9.0 had a high rate of HCO₃⁻-dependent O₂ evolution that was only slightly inhibited by SHAM. It appears that SHAM inhibited a DIC pump which utilizes external CO₂, but did not inhibit an external HCO₃⁻ transporter at pH 9.

Concentration curves for SHAM inhibition of photosynthesis, respiration, and DIC uptake are only of experimental value. SHAM is relatively insoluble in water, and addition of 1 to 5 mM SHAM from a stock solution of 0.45 mM in ethanol is an operational measurement; the amount of effective SHAM that remained in aqueous solution (not in suspension) is difficult to assess, but it is less than that added. In evaluating the relative SHAM concentration, we have compared its effect on photosynthesis (Fig. 3B) and respiration at similar concentrations (13). With CO₂-grown Chlamydomonas the addition of up to 5 mM SHAM did not inhibit photosynthetic oxygen evolution (Fig. 3A). Both in CO₂-grown and air-adapted cells with high DIC levels, the inhibition of ¹⁴CO₂-fixation by addition of 5 mM SHAM was only about 10% (Fig. 3B). With air-adapted cells at pH 7 or 7.5 on limiting DIC, O₂ evolution was progressively inhibited by increasing concentration of SHAM with a Kᵣ of about 2.5 mM SHAM. This inhibition was reversed by high DIC concentrations (Fig. 1; Table 1). These results suggest that SHAM did not inhibit the photosynthetic capability of the cells, but rather the SHAM inhibition of photosynthesis was due to reduced DIC uptake. Nevertheless, to avoid other possible effects from SHAM, only concentrations of 2 to 3 mM were added with Chlamydomonas or Dunaliella cells, although these levels did not completely inhibit the CO₂ pump. In these experiments, similarly, concentrations of SHAM partially inhibited respiration by Chlamydomonas (13), also with a Kᵣ of about 2.5 mM. With Scenedesmus, concentrations of SHAM above 2 mM became inhibitory to photosynthetic oxygen even at high DIC levels, and the cells became brownish in color (results not shown). Below 2 mM SHAM, O₂ evolution by Scenedesmus with excess DIC was not significantly inhibited. Thus, only 1.5 mM SHAM was used in experiments with Scenedesmus. SHAM (1-5 mM) did not inhibit isolated periplasmic CA from Chlamydomonas, nor did it inhibit the CA activity measured with the whole cells (results not shown).

Up to 1 mM n-Propylgallate did not inhibit the DIC pump in Chlamydomonas or CO₂ fixation. Tetraethyl thiuram disulfide (disulfiram) at 10 μM inhibited CO₂ fixation and could not be tested as a DIC pump inhibitor. Although these toxic compounds inhibit the alternative respiration, they are not considered to be as specific inhibitors as SHAM.

Effect of SHAM on ¹⁴C-inorganic carbon uptake and ¹⁴CO₂-fixation

The effect of SHAM on DIC-uptake by air-adapted cells of Chlamydomonas was measured by incubating the cells for 0.5 to 2 min with SHAM and low concentrations of [¹⁴C]HCO₃⁻ (150 μM HCO₃⁻), which required a DIC pump for rapid accumulation. Total DIC uptake would be the sum of ¹⁴C fixation and DIC accumulation in the cells. DIC accumulation was calculated by subtracting acid stable radioactivity from the total ¹⁴C carbon uptake. With our experimental conditions, total DIC accumulation was maximized after 1 to 2 min (Fig. 4) and varied between 5 to 8 mM. Consequently, most rates of DIC accumulation were measured over only 60 to 120 s. In Chlamydomonas 2 mM SHAM inhibited by 70 and 80% the ¹⁴C accumulation in 1 min at pH 7.0 (Fig. 4) or 7.5 (data not shown). Higher concentrations of SHAM (4 mM) inhibited uptake more than 90% at both pH. In 1 to 2 min of photosynthesis, which did not reach Vₘᵢₓ, the total ¹⁴CO₂ fixation at pH 7.0 or 7.5 was inhibited 45% with 2 mM SHAM and about 90% with 4 mM SHAM. Most of the inhibition of ¹⁴CO₂ fixation was due to reduced carbon accumulation and not due to impaired fixation ability (Fig. 3), because the rate of CO₂ fixation approached Vₘᵢₓ with higher NaHCO₃ concentrations.

With Scenedesmus, the DIC accumulation was inhibited about 50 to 60% by 1.5 mM SHAM in the cells adapted to air at pH 7.2 and tested at pH 7.2 (Fig. 5A) or about 80% when tested at pH 9.0 (Fig. 5B). These cells air-adapted at pH 7.2 had a well developed CO₂ pump, but the HCO₃⁻ pump was less developed. The Scenedesmus cells, adapted at pH 9.0 to activate the HCO₃⁻ pump, when tested at pH 7.2 were inhib-
Both pH uptake. Inhibited by 50% by 1.5 mM SHAM (Fig. 5C). The same cells air-adapted at pH 9, when tested at pH 9, took up substantial DIC by their HCO\textsuperscript{3-} transporter (28), and 1.5 mM SHAM hardly inhibited this accumulation. These results are similar to those obtained by measuring photosynthetic oxygen evolution. SHAM inhibited the DIC pump, that had been formed at pH 7.2 or 9.0 for external CO\textsubscript{2}, but SHAM was not a good inhibitor of the HCO\textsuperscript{3-} pump when the algal DIC pumps were activated and tested at pH 9. At alkaline pH, Scenedesmus predominantly uses a HCO\textsuperscript{3-} pump, but cells air-adapted at pH 9.0 and tested at pH 7.2 had a CO\textsubscript{2} pump to concentrate available CO\textsubscript{2} at either pH (28), and this CO\textsubscript{2} uptake was inhibited by SHAM. At pH 9.0, inorganic carbon was in the form of HCO\textsuperscript{3-}, and its uptake was not inhibited by SHAM.

\[ ^{14}\text{C} \text{O}_2 \text{ fixation was inhibited about 50\% by 1.5 mM SHAM by air-adapted Scenedesmus cells at pH 7.2 when tested at both pH 7.2 and 9.0 (Table II). For Scenedesmus cells air-adapted at pH 9 and tested at pH 7.2, the SHAM inhibition of CO}_2 \text{ fixation seems to be associated with its inhibition of CO}_2 \text{ uptake. Consequently, at pH 9.0 photosynthesis by these cells was inhibited only 20\% by SHAM. SHAM inhibition of }^{14}\text{C} \text{O}_2 \text{ fixation at pH 7 would appear to be due to SHAM inhibition to a decrease in DIC accumulation. At alkaline pH 9.0, SHAM neither inhibited DIC accumulation nor }^{14}\text{C} \text{O}_2 \text{-fixation.}]

\textbf{Bicarbonate Uptake by Chloroplasts Isolated from Dunaliella was not Inhibited by SHAM}

Isolated intact chloroplasts from air-adapted \textit{Chlamydomonas} (20) and \textit{Dunaliella} (14) cells, but not CO\textsubscript{2}-grown cells, accumulate DIC. When tested with Dunaliella chloroplasts, this accumulation from the medium was vanadate sensitive, as if an ATPase was involved (14), and thus we proposed that the chloroplast DIC pump involved an ATP/HCO\textsuperscript{3-} transporter. The chloroplast DIC uptake process was not inhibited by SHAM (Fig. 6), whereas SHAM did inhibit CO\textsubscript{2} uptake by whole Dunaliella cells at pH 7.0 and 7.5 (Table I) similarly to \textit{Chlamydomonas} and Scenedesmus. These results are consistent with SHAM inhibition of CO\textsubscript{2} uptake but not HCO\textsuperscript{3-} uptake by the whole cells or chloroplasts.

\textbf{DISCUSSION}

Salicylhydroxamic acid (SHAM) at 1 to 2 mM inhibited DIC accumulation by three algae, \textit{Chlamydomonas}, \textit{Scene-
Table II. Effect of SHAM on the $^{14}$CO$_2$ Fixation (Acid Stable Radioactivity) by Chlamydomonas and Scenedesmus

<table>
<thead>
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<th>Organism</th>
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<th>4 mM SHAM</th>
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<td>C. reinhardtii in 50 mM Hepes buffer at pH 7.0</td>
<td>63</td>
<td>52</td>
<td>4</td>
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<tr>
<td>S. obliquus in 20 mM phosphate buffer at assay pH</td>
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<tr>
<td>9.0</td>
<td>31</td>
<td>80</td>
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* Assays with Scenedesmus plus 2 and 4 mM SHAM were not run due to SHAM toxicity; these concentrations of added SHAM did not inhibit photosynthesis by Chlamydomonas with 2 mM NaHCO$_3$.

Figure 6. Lack of SHAM inhibition of bicarbonate uptake by Dunaliella chloroplasts. These chloroplasts were isolated and tested for DIC uptake as previously described (14): 150 μM [14C]NaHCO$_3$ and 5 mM SHAM were used. Chloroplasts from air-adapted cells (O, C); chloroplasts from CO$_2$-grown cells (A, S). Closed symbols, control without SHAM; open symbols, with 5 mM SHAM.

adapted cells. However, vanadate, an ATPase inhibitor, inhibited HCO$_3^-$ uptake at pH 9 by Scenedesmus (28) or by isolated chloroplasts (14). Thus, SHAM and vanadate appear to be two inhibitors of DIC uptake that differentiate between CO$_2$ uptake and HCO$_3^-$ uptake. Interpreting our results is complicated by the fact that both pumps coexist and overlap between pH 6 and 8.3 where DIC is a mixture of CO$_2$ and HCO$_3^-$. In addition, most solutions generally have initially enough dissolved CO$_2$ even at pH 8.3 to sustain considerable O$_2$ evolution until the CO$_2$ is reduced below Kd,CO$_2$. These problems were managed by using solutions nearly DIC free and by removing the endogenous DIC in a preillumination period of 1 to 2 min until O$_2$ evolution ceased (see procedure and Fig. 1 of ref. 28). Nevertheless, further additions, such as a SHAM solution, carried in additional CO$_2$ which in turn had to be depleted. Another problem was that the CO$_2$ pump in Scenedesmus seems to be more active or dominated over the HCO$_3^-$ pump as long as there were significant levels of CO$_2$.

SHAM has been used extensively as an inhibitor of the mitochondrial alternative oxidase in plants (9, 26). SHAM also inhibits respiration in algae, where the location and mechanism of its action are not characterized (13). SHAM inhibition of active CO$_2$ accumulation by algae could be due to a role of the alternative oxidase system in CO$_2$ uptake or to a nonrelated SHAM inhibition of a respiratory component of the DIC pumps. The need for energy for DIC pumps by algae is widely recognized (16, 24). The function of the alternative oxidase in mitochondria is not known other than to dissipate excess energy without generating ATP. Since the operation of DIC pumps requires energy, and since SHAM inhibition of the alternative oxidase in the mitochondria should not inhibit ATP generation, it seems unlikely that SHAM inhibition of the CO$_2$ pump should be due to reduced mitochondrial alternative oxidase activity. Thus, other hy-
hypothesis are needed to explain SHAM inhibition of the CO₂ pump. This type of respiration, involving quinol oxidation, might be in other membranes to create ion exchange gradients. The large increase in pH of the algal medium when CO₂ is taken up indicates a requirement for neutralization of the OH⁻ when HCO₃⁻ is converted to CO₂ and OH⁻. This conversion is usually facilitated by periplasmic CA (19), and indeed acetazolamide, a CA inhibitor, reduced both CO₂ uptake and alkalinization of a neutral medium (results not shown). However, SHAM did not inhibit the partially isolated periplasmic CA. In nature during photosynthesis the large volume of bathing medium may wash away the hydroxyl ion or the OH⁻ may be used to convert a HCO₃⁻ to CO₂, which is precipitated as CaCO₃ (7). Within the periplasmic space, there may also be a respiratory requirement for an H⁺ to titrate HCO₃⁻ to CO₂ and water or to neutralize the OH⁻, and in some manner SHAM is inhibiting this process.

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LITERATURE CITED