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Alternative Methods of Photosynthetic Carbon Assimilation in Marine Macroalgae[1]

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ABSTRACT

Two green macroalgae, *Codium decorticatum* and *Udotea flabellum*, differ photosynthetically. *Codium* had high O_2-sensitive, and *Udotea* low O_2-insensitive, CO_2 compensation points; *Codium* showed a Warburg effect at seawater dissolved inorganic carbon levels and had photorespiratory CO_2 release, whereas *Udotea* did not. Seawater dissolved inorganic carbon levels did not saturate photosynthesis. For *Codium*, but not *Udotea*, the Warburg effect was increased by ethoxyzolamide, a carbonic anhydrase inhibitor, at high but not low pH. Isolated chloroplasts from both macroalgae showed a Warburg effect that was ethoxyzolamide-insensitive. In both macroalgae, chloroplastic and extrachloroplastic carbonic anhydrase activity was present. P-enolpyruvate carboxykinase (PEPCK) carboxylating activity in *Udotea* extracts was equivalent to that of ribulose bisphosphate carboxylase, and enzyme activities for C_4 acid metabolism and P-enolpyruvate regeneration were sufficient to operate a limited C_4-like system. In *Udotea*, malate and aspartate were early-labeled photosynthetic products that turned over within 60 seconds. Photorespiratory compounds were much less labeled in *Udotea*. Low dark fixation rates ruled out Crassulacean acid metabolism. A limited C_4-like system, based on PEPCK, is hypothesized to be the mechanism reducing photorespiration in *Udotea*. *Codium* showed no evidence of photosynthetic C_4 acid metabolism. Marine macroalgae, like terrestrial angiosperms, seem to have diverse photosynthetic modes.

In the three divisions of marine macroalgae, Rhodophyta (reds), Phaeophyta (browns), and Chlorophyta (greens), RuBPCO[2] has been reported to be a major carboxylase, and labeling studies point to a functional PCR cycle (6, 22, 24). Photorespiration measurements and labeling of glycine and serine suggest that the PCO cycle also operates, and, furthermore, all three divisions contain species which exhibit O_2 inhibition of photosynthesis or the Warburg effect and high Γ values (5–7, 22, 24, 28). This has led to the general conclusion that marine macroalgae are C_3 (5), not C_4 plants (24). However, recent observations that many marine macroalgae have low Γ values and no Warburg effect at seawater DIC levels (4, 6, 19) are inconsistent with C_3 photosynthesis.

Two methods of reducing the Warburg effect are well-established. In terrestrial C_4 plants, it is by the C_4 acid cycle in conjunction with Kranz anatomy (11). Among unicellular photoautotrophs, active DIC accumulation mechanisms operate, usually in association with CA activity (2, 25). Both systems serve to concentrate CO_2 at the site of fixation by RuBPCO, thus suppressing O_2 effects and subsequent photorespiration.

There is evidence that many, but not all, marine macroalgae use HCO_3^- ions for photosynthesis (4, 6, 10, 22, 24) and that CA is required (4, 10, 22). Thus, the possibility that they concentrate CO_2, in a manner comparable to unicellular organisms, has been broached (4, 6, 10); though there are no direct measurements of internal DIC levels to substantiate this hypothesis.

Enzymes of C_4 acid metabolism and C_4 acid labeling during photosynthesis occur in some macroalgae (6, 22–24). In the case of brown macroalgae and marine diatoms, substantial β-carboxylation of PEP in the light and dark has been reported (20, 24). The enzyme responsible is PEPCK acting in a carboxylating mode, not PEPC which occurs in terrestrial C_4 plants (6, 20, 22, 24). Dark fixation and diel titratable acidity changes associated with the PEPCK activity (22, 24) are small, and a CAM-like system in brown macroalgae has been discounted (21). The operation of C_4-like photosynthesis in marine macroalgae (23) is also questionable, as crucial data to show rapid C_4 acid turnover in the light is lacking, and no linkage has been demonstrated between C_4-like gas exchange characteristics and C_4 biochemistry.

In this study, we report on two marine macroalgae in the Chlorophyta, which, though closely related, exhibit very different gas exchange and biochemical characteristics during photosynthesis. *Udotea*, in contrast to *Codium*, has little photorespiration or Warburg effect and exhibits PEPCK-mediated C_4 acid metabolism in the light.

MATERIALS AND METHODS

Plant Materials. *Codium decorticatum* (Woodward) Howe (division Chlorophyta) was collected during late winter and early spring from intertidal rocks at Matanzas Inlet and Turtle Mound on the Atlantic coast of Florida. *Udotea flabellum* (Ellis and Solander) Lamouroux (division Chlorophyta) was found in spring, summer, and autumn rooted in sand at a depth of 2 to 4 m off Key West or at the mouth of the Homosassa River in the Gulf of Mexico, Florida. After collection, the thalli were washed and cleaned of epiphytes. The plants were maintained in growth chambers in 20 L aquaria containing filtered, aerated seawater under a 20°C/12-h photoperiod with a quantum irradiance of 150 μmol m^{-2} s^{-1} (400–700 nm) and a 16°C scotoperiod. The seawater was changed twice weekly, and every 2 weeks the plants were placed overnight in a 25% v/v Hoagland nutrient solution made up with seawater.

Gas Exchange Analyses. Net photosynthetic rates were determined as O_2 evolution with a Rank electrode system at 21% and

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[2] Abbreviations: RuBPCO, ribulose-1,5-bisP carboxylase-oxygenase; CA, carbonic anhydrase; DIC, dissolved inorganic carbon (CO_2 + HCO_3^-); EZ, ethoxyzolamide; Γ, CO_2 compensation point; IRGA, infrared gas analysis; OAA, oxaloacetate; PCR, photosynthetic carbon reduction; PCO, photorespiratory carbon oxidation; PEP, P-enolpyruvate; PEPC, P-enolpyruvate carboxylase; PEPCK, P-enolpyruvate carboxykinase; TCA, tricarboxylic acid.

1% gas phase O_2 (equivalent to 220 and 11 μM O_2, respectively) in synthetic seawater buffered at pH 8.0 with 10 mM Tris-HCl, and containing, unless otherwise stated, 2 mM DIC. Gas-phase equilibrium Γ values and apparent photorespiration, measured as CO_2 evolution into CO_2-free air in the light, were determined with an ADC (Analytical Development Corp., England) 225 MK3 IRGA at 21% and 1% O_2. Dark respiration and net photosynthetic rates were also measured with this system at ambient (11 μM) CO_2. For the IRGA studies, plants were incubated in DIC-free synthetic seawater buffered with 10 mM Mes-NaOH at pH 5.5 and were sparged with the appropriate gas mixtures of N_2, O_2, and CO_2. Gas exchange measurements were made at 20 and 23°C for *Codium* and *Udotea*, respectively, and a saturating quantum irradiance of 300 μmol m^{-2} s^{-1} (400–700 nm).

The effects of EZ, a membrane-permeable inhibitor of CA, on net photosynthesis at 2 mM DIC and 21 and 1% O_2 were determined in the O_2 electrode system. Before analysis, thalli were incubated for 60 min in synthetic seawater buffered as above at pH 5.0 or 8.0, with or without 100 μM EZ.

Chloroplast Isolation and Assay. Chloroplasts were isolated from *Codium* and *Udotea* in the light by the methods of Cobb (8) and Bidwell *et al.* (3), respectively. *Codium* chloroplast photosynthesis was determined as O_2 evolution in a solution of 10 mM Tris-HCl, 0.8 M sucrose, and 15 mM $MgCl_2$ at pH 7.8. For *Udotea* chloroplasts, rates were determined in a medium of 0.6 M mannitol, 5 mM Hepes-KOH, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM KH_2PO_4, 5 mM PPi, and 5 mM 3-P-glycerate at pH 7.5. In both cases, the assay medium contained 30 μg Chl ml^{-1} and 10 mM $NaHCO_3$, and measurements were made at 21 and 1% O_2. The rates were determined after a 20 min incubation with or without 100 μM EZ. The same temperature and quantum irradiance used for measuring thalli photosynthetic rates were used for the chloroplasts.

Enzyme Extractions. One g fresh weight of plant material was ground in 4 ml of extraction medium in a Ten Broeck homogenizer at 4°C. The basic extraction medium for all the enzymes, except glycolate oxidase, glycolate dehydrogenase, and CA, consisted of 0.2 mM EDTA, 5 mM DTT, 10 mM $MgCl_2$, 2% w/v PVP-40, and 200 mM Hepes-KOH (pH 7.0) or Tris-HCl (pH 8.0). The PEP carboxylating enzymes were extracted at pH 7.0, and all others at pH 8.0. $MnCl_2$ replaced $MgCl_2$ for the PEPCK and NAD/NADP malic enzymes. The aminotransferases were extracted with added pyridoxal phosphate (20 μg ml^{-1}), while 5 mM D-isoascorbate was included for the 3-P-glycerate and P-glycolate phosphatases. For pyruvate Pi dikinase, the plant material was homogenized at room temperature in the light under O_2-free conditions. The procedure of Graham and Smillie (13a) was used for the extraction of CA and that of Frederick *et al.* (13) for glycolate oxidase and dehydrogenase.

Enzyme Assays. All enzymes were assayed at 30°C. RuBPCO was activated and assayed following the method of Vu *et al.* (31), and PEPC by the method of Van *et al.* (30). PEPCK (carboxylating mode) was assayed radiochemically (20) and spectrophotometrically with NADH; this enzyme was assayed also in the decarboxylating mode as described by Hatch (14). Adenylate kinase (16), aspartate and alanine aminotransferases (15), the malate dehydrogenases, and malic enzymes were assayed spectrophotometrically (18). Pyruvate Pi dikinase was assayed radiochemically (18). Glycolate oxidase and dehydrogenase were measured spectrophotometrically by following the decrease in absorbance at 600 nm due to the anaerobic reduction of 2,6-dichlorophenolindophenol in the presence of glycolate, and L- or D-lactate (13). The rate of Pi formation was used to determine the activities of 3-P-glycerate and P-glycolate phosphatases (26), and pyrophosphatase (16). CA activity was ascertained by the method of Hogetsu and Miyachi (17), and the enzyme units (EU) were calculated using the formula: $EU = ([T_o - T]/T)\ 10$, where T_0 = the rate of nonenzymic hydration, and T_o = the rate of enzymic hydration. All enzyme analyses were performed in triplicate.

Fixation of ^{14}C. One cm^2 sections of *Udotea* blades and 2 cm long *Codium* tips were incubated for 1 h in 4 ml of DIC-free synthetic seawater and were buffered at pH 8.0, under low light (100 μmol m^{-2} s^{-1}) at 22°C, to deplete endogenous DIC reserves. They were then allowed to photosynthesize at optimum temperature and saturating quantum irradiance for 10 or 60 s in synthetic seawater containing 2 mM [^{14}C]$NaHCO_3$ (6.25 Ci mol^{-1}). After the ^{14}C pulse, the algal segments were immediately rinsed and frozen in liquid N_2 or were rinsed and placed in the medium with unlabeled $NaHCO_3$ for varying chase periods before freezing. Dark ^{14}C fixation was determined in a similar medium containing 2 mM [^{14}C]$NaHCO_3$ (1.25 Ci mol^{-1}) over a 5 min period.

Analyses of ^{14}C Fixation Products. Samples frozen in liquid N_2 were extracted (27), and aliquots were taken for total ^{14}C incorporation and Chl measurements. After removal of the lipid and insoluble components, a combination of ion exchange and TLC was used to separate and analyze the labeled products (27). The recovery of ^{14}C was 92%. Labeled [^{14}C]fructose-1,6-bisP, malate, 3-P-glycerate, and glycolate, in addition to unlabeled aspartate, alanine, glutamate, glycine, and serine, which were detected by ninhydrin spray, were used as standards.

Chl Determinations. Chl was determined by the method of Arnon (1).

RESULTS

Gas Exchange. Table I shows photosynthetic and respiratory gas exchange responses for *Codium* and *Udotea* determined in the IRGA system. The Γ for *Codium* at 21% O_2 was high, but at 1% O_2 it decreased substantially, though not to zero. *Codium* photosynthesis was inhibited by 21% O_2, and CO_2 evolution into CO_2-free air in the light (a measure of apparent photorespiration) was threefold greater at 21% than at 1% O_2. In contrast, the Γ in *Udotea* was low and was unaffected by O_2; net photosynthesis showed only a slight inhibitory effect of O_2; and apparent photorespiration was negligible. Dark respiration for *Codium* was lower than for *Udotea*; the rates were 19.7 and 14.4% of net photosynthesis, respectively.

Net photosynthetic rates as a function of DIC concentration were determined for *Codium* at 21 and 1% O_2 (Fig. 1). *Codium* photosynthesis was not fully saturated at the usual seawater DIC concentration of 2 mM and pH 8.0. The apparent K_m (DIC) for photosynthesis was 0.67 mM. At all DIC levels, 21% O_2 exerted a marked inhibitory effect on *Codium* photosynthesis (Fig. 1). The higher DIC concentrations partially reversed the inhibition, but did not eliminate it, even at 10 mM DIC.

As with *Codium*, the photosynthesis of *Udotea* required higher DIC levels than those in seawater to achieve saturation (Fig. 2), and the apparent K_m (DIC) was about 2.0 mM. In contrast to *Codium*, there was little Warburg effect in *Udotea* at DIC levels above 1 mM.

The effects of EZ and O_2 on net photosynthesis at high and low pH are seen in Table II. Photosynthesis by the thalli of both *Codium* and *Udotea* was inhibited by EZ at pH 8 and 2 mM DIC, with *Codium* being far more sensitive than *Udotea*. Furthermore, the presence of EZ doubled the inhibitory effect of O_2 on *Codium* photosynthesis but did not increase it in *Udotea*. At pH 5, for each alga the O_2 inhibition was similar to that observed at pH 8. EZ had little effect on photosynthesis or O_2 inhibition at low pH.

Similar studies were performed on chloroplasts isolated from the macroalgae (Table II). In the absence of EZ, O_2 inhibited the photosynthesis of chloroplasts from both macroalgae to a greater

Table I. *Gas Exchange Responses for Two Marine Macroalgae*

Measurements were performed in seawater at pH 5.5 using the IRGA system. Photosynthetic and dark respiration rates were measured at 11 μM CO_2. Data represent the mean of three replicates ± SD.

Macroalga	O_2 Level	Γ	Net Photosynthetic Rate	Inhibition by CO_2	CO_2 Evolution into CO_2-Free Air in the Light	Dark CO_2 Evolution
	%	μl CO_2 L^{-1}	μmol CO_2 mg^{-1} Chl h^{-1}	%	μmol CO_2 mg^{-1} Chl h^{-1}	
Codium	21	56	6.1 ± 0.6	21	1.2 ± 0.04	1.2 ± 0.2
	1	20	7.7 ± 0.8		0.4 ± 0.01	
Udotea	21	9	12.5 ± 3.1	7	0.1 ± 0.03	1.8 ± 0.3
	1	7	13.4 ± 2.9		0.1 ± 0.04	

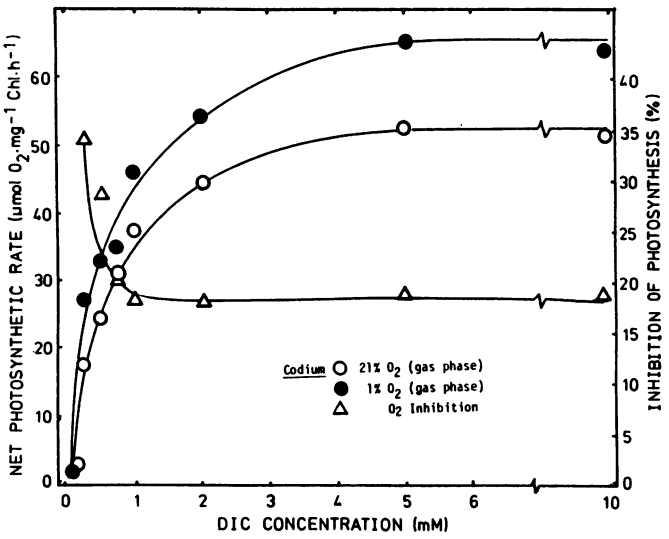

FIG. 1. Net photosynthetic rates of *Codium* thalli as a function of the total dissolved inorganic carbon concentration at 21% and 1% O_2 (gas phase), and the percent O_2 inhibition of photosynthesis. Each data point represents the mean of three replicates.

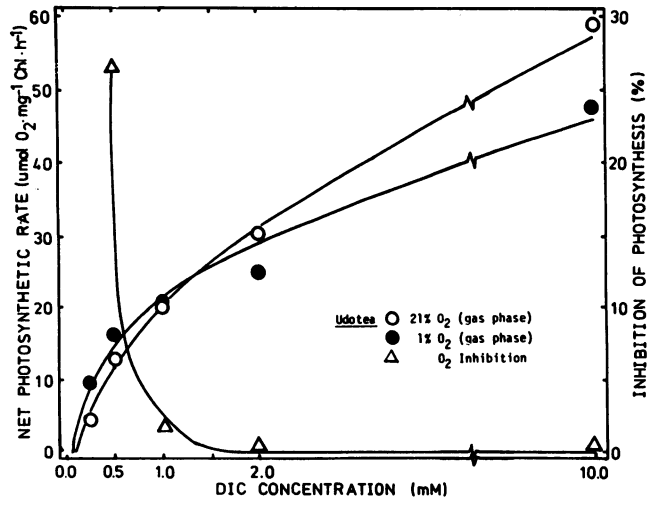

FIG. 2. Net photosynthetic rates of *Udotea* thalli as a function of the total dissolved inorganic carbon concentration at 21% and 1% O_2 (gas phase), and the percent O_2 inhibition of photosynthesis. Each data point represents the mean of three replicates.

extent than it did of their thalli, even though in the chloroplast experiments the DIC level (10 mM) was higher. In the presence of 100 μM EZ, the photosynthetic rate of *Codium* chloroplasts was approximately halved, while for *Udotea* chloroplasts it was halted. Unlike the thallus results, at high pH EZ did not greatly increase the O_2 inhibition of *Codium* chloroplast photosynthesis.

Enzyme Activities. Table III presents data for the CA activity associated with various cellular fractions of the two macroalgae. In the same cellular fractions, enzyme activities were similar for both algae. There was little CA activity associated with the pellet fractions from the thalli or chloroplasts; whereas the soluble fractions exhibited substantial activity. The intact chloroplasts of both macroalgae had CA activity associated with them, even after being well washed.

Table III also shows the activities of three carboxylases in crude extracts. RuBPCO appeared to be a primary carboxylating enzyme, but it is notable that in *Udotea* its activity was rivaled by PEPCK. Activities of PEPCK measured before and after the biweekly nutrient exposure were not significantly different (data not shown). In *Codium* extracts, RuBPCO activities surpassed the *in vivo* light- and DIC-saturated photosynthetic rates, as did both RuBPCO and PEPCK in *Udotea*. Extracts from both macroalgae had low PEPC activities, at only 1 to 2% of the RuBPCO levels.

The functional requirements of PEPCK (carboxylating) activity in *Udotea* extracts were examined to ensure that PEPCK was indeed being measured, and not the residual activity of some other carboxylase. From Table IV it is clear that Mn^{2+}, ADP, HCO_3^-, and PEP were essential reaction components, and ADP and PEP could not be replaced by ATP or pyruvate, respectively. The addition of glucose and hexokinase, as an ATP trap to eliminate potential interference by pyruvate kinase and pyruvate carboxylase, did not markedly decrease the activity.

The activities of enzymes associated with C_4 acid metabolism, the regeneration of PEP, and photorespiration were measured in the algal extracts (Table V). Enzymes of C_4 acid metabolism were substantially higher in activity in *Udotea* extracts than in *Codium*, as were those required for PEP regeneration, with the exception of pyrophosphatase. Pyruvate Pi dikinase activity in *Udotea* was high enough to support the photosynthetic rate. There were no detectable activities of decarboxylases in *Codium* extracts. Similarly, NADP-malic enzyme was not detected in *Udotea*, and only trace levels of the NAD form were seen. However, the decarboxylating activity of PEPCK in *Udotea* extracts was nearly as high as the carboxylating. Of the enzymes associated with the PCO cycle, P-glycolate phosphatase and glycolate dehydrogenase (soluble fraction) activities were found in *Codium*, but only 3-P-glycerate phosphatase was detected in *Udotea*.

Photosynthetic Products. A short (10 s) pulse of *Udotea* thallus with $^{14}CO_2$ resulted in 25% of the label incorporated into the C_4 acids malate and aspartate, 23% in 3-P-glycerate, and 37% into sugar-P. To further investigate the ^{14}C incorporation patterns in the light, thalli of both macroalgae were subjected to a 60 s $^{14}CO_2$ pulse followed by a $^{12}CO_2$ chase. Figure 3 shows that for both algae after the pulse (zero chase time) approximately 20 to 30% of the label was in 3-P-glycerate. At this point, with *Codium*

Table II. *Effects of 100 μM Ethoxyzolamide (EZ) and O_2 on the Photosynthetic Rates of Thalli and Isolated Chloroplasts of Codium and Udotea*

Thalli and chloroplasts were measured at 2 and 10 mM dissolved inorganic carbon, respectively. Data represent the mean of three replicates ± SD.

Macroalga	pH	Treatment	Net Photosynthetic Rate at 21% O_2	O_2 Inhibition (21% *versus* 1%)	EZ Inhibition
			μmol O_2 mg^{-1} Chl h^{-1}	%	%
Codium					
Thalli	5.0	Control	30.7 ± 6.9	30	
		EZ	30.1 ± 0.8	31	2
	8.0	Control	62.7 ± 5.8	26	
		EZ	7.3 ± 2.8	58	88
Chloroplasts	7.8	Control	20.4 ± 2.1	40	
		EZ	8.7 ± 1.5	49	57
Udotea					
Thalli	5.0	Control	22.0 ± 2.1	9	
		EZ	21.3 ± 2.0	7	3
	8.0	Control	35.3 ± 18.7	15	
		EZ	21.7 ± 11.5	8	39
Chloroplasts	7.8	Control	35.1 ± 3.7	25	
		EZ	0		100

Table III. *Activities of Carbonic Anhydrase and Three Carboxylases in Extracts of Two Marine Macroalgae*

The activities of the carboxylases were determined in thallus homogenates. Data represent the mean of three replicates ± SD.

Enzyme	Enzyme Activity	
	Codium	*Udotea*
	enzyme units mg^{-1} Chl	
Carbonic anhydrase		
Thallus		
Homogenate	161.6 ± 12.2	130.2 ± 17.1
Supernatant	194.2 ± 5.2	187.0 ± 20.3
Pellet	0	0
Chloroplast		
Intact	340.6 ± 60.7	374.6 ± 79.5
Homogenate	207.9 ± 26.5	587.5 ± 92.4
Supernatant	408.8 ± 59.9	352.8 ± 52.7
Pellet	38.5 ± 3.0	0
	μmol mg^{-1} Chl h^{-1}	
RuBPCO	100.8 ± 2.7	79.1 ± 2.2
PEPC	1.3 ± 0.1	2.4 ± 0.1
PEPCK (carboxylating)	0.9 ± 0.1	89.3 ± 2.1

Table IV. *Functional Requirements of PEPCK Assayed in Udotea thalli Extracts*

Data represent the mean of three replicates ± SD.

Reaction Mixture Components	PEPCK Activity
	μmol NADH mg^{-1} Chl h^{-1}
Complete	89.3 ± 2.1
− $MnCl_2$	ND[a]
− ADP	ND
− HCO_3^-	4.2 ± 0.8
− PEP	ND
− PEP + pyruvate	1.0 ± 0.3
− $MnCl_2$ − ADP + $MgCl_2$	ND
− ADP + ATP	ND
+ glucose + hexokinase	75.7 ± 3.4

[a] Not detected.

Table V. *Activities of Enzymes Associated with C_4 Acid Metabolism, P-enolpyruvate Regeneration, and Photorespiration in Codium and Udotea*

Data represent the mean of three replicates ± SD.

Enzyme	Enzyme Activity	
	Codium	*Udotea*
	μmol mg^{-1} Chl h^{-1}	
C_4 acid metabolism		
NAD malate dehydrogenase	1052 ± 157	3584 ± 338
NADP malate dehydrogenase	ND[a]	7.0 ± 0.1
NAD malic enzyme	ND	trace
NADP malic enzyme	ND	ND
PEPCK (decarboxylating)	ND	66.0 ± 2.9
Aspartate aminotransferase	350 ± 43.2	821 ± 61.4
Alanine aminotransferase	90.4 ± 20.1	135 ± 0.7
PEP regeneration		
Pyruvate Pi dikinase	9.8 ± 1.1	30.4 ± 2.8
Pyrophosphatase	1236 ± 113	222 ± 17.1
Adenylate kinase	5.6 ± 1.1	174 ± 16.2
Photorespiration		
P-glycolate phosphatase	80.1 ± 12.9	ND
P-glycerate phosphatase	ND	64.0 ± 17.2
Glycolate oxidase	ND	ND
Glycolate dehydrogenase	4.7 ± 0.8	ND

[a] Not detected.

there was essentially no label in malate and aspartate. However, in *Udotea* over 16% of the ^{14}C was located in these C_4 acids, and there was a rapid decline in the labeling of C_4 acids during the first 60 s of the chase. This was accompanied by a rise in 3-P-glycerate labeling between 30 and 60 s into the chase, after which it declined to a steady state level. In *Codium*, during the chase period the 3-P-glycerate declined to 4%, and although the C_4 acid label increased, it remained a relatively minor component.

As can be seen in Figure 4, at the end of the 60 s pulse (zero chase time) the bulk of the label was in phosphorylated compounds (including 3-P-glycerate) with much less in the insoluble and neutral compounds. During the chase period, there was a decline in label in the phosphorylated compounds with an accompanying increase in the insoluble and neutral fractions. This pattern was similar for both macroalgae, although the label in insoluble and neutral compounds at the start of the chase was higher for *Udotea*.

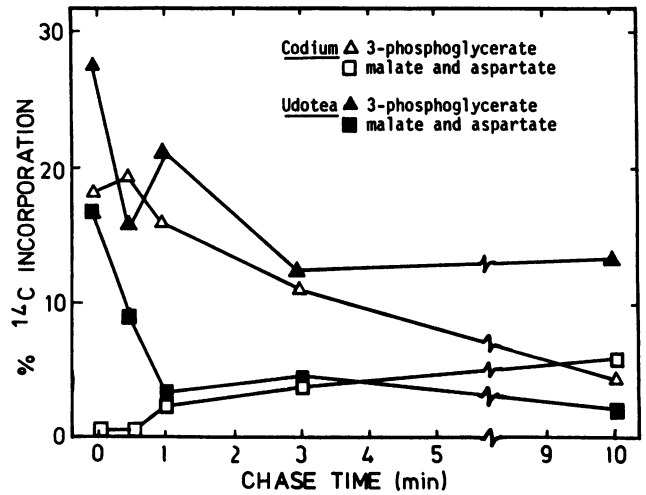

FIG. 3. Distribution of ^{14}C in C_4 acids and 3-P-glycerate of *Codium* and *Udotea* thalli during a pulse-chase experiment in the light. The zero time point represents the start of the ^{12}C-chase following a 60 s ^{14}C-pulse. Incorporation is expressed as a percentage of the total ^{14}C incorporated. The $^{14}CO_2$ fixation rates were 43.1 ± 17.8 and 15.5 ± 2.7 μmol mg^{-1} Chl h^{-1} for *Codium* and *Udotea*, respectively.

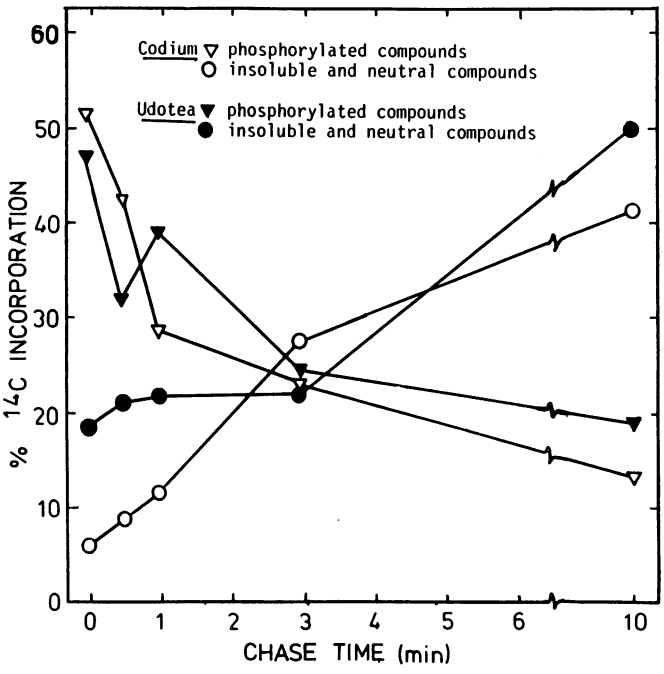

FIG. 4. Distribution of ^{14}C among the photosynthetic compounds of *Codium* and *Udotea* thalli during a pulse-chase experiment in the light. The zero time point represents the start of the ^{12}C-chase following a 60 s ^{14}C-pulse. Incorporation is expressed as a percentage of the total ^{14}C incorporated. The $^{14}CO_2$ fixation rates were as in Figure 3.

Table VI shows the ^{14}C incorporation patterns in PCO cycle compounds, alanine/glutamate, and the lipid fraction after a 60 s pulse followed by an unlabeled chase. *Codium* showed a higher percent label in the PCO cycle compounds glycolate, glycine, and serine than did *Udotea*, especially at the end of the pulse, though after 10 min the difference was less marked. There was greater initial ^{14}C incorporation in the alanine/glutamate fraction of *Codium* than *Udotea*, but with longer chase periods the percentages became similar. For *Udotea*, alanine labeling remained constant, being 3.2, 4.1, 3.8, and 3.6% at 0, 1, 3, and 60 min into the chase, respectively. Label in the lipid fraction was always greater with *Codium* than *Udotea*.

Table VI. *Time Course of ^{14}C Incorporation into Compounds of the Photorespiratory Carbon Oxidation Cycle, Basic, and the Lipid Fractions for Codium and Udotea*

Zero time represents the start of the unlabeled chase after a 60 s pulse with ^{14}C. Fixation rates were as in Figure 3.

Macroalga	Chase Time	^{14}C Incorporation		
		Glycolate, Glycine, Serine	Alanine/ Glutamate	Lipid
	min		%	
Codium	0	7.1	11.1	9.3
	3	9.3	10.2	12.6
	10	6.5	7.8	12.4
Udotea	0	1.0	3.9	6.4
	3	5.1	9.4	7.6
	10	4.6	8.7	5.3

Table VII. *Light and Dark $^{14}CO_2$ Fixation Rates for Thalli of Codium and Udotea*

Measured at 2 mM dissolved inorganic carbon pH 8.0, and 21% O_2. Data represent the mean of three replicates ± SD.

Treatment	Fixation Rate	
	Codium	*Udotea*
	μmol mg^{-1} Chl h^{-1}	
Light	41.2 ± 3.3	20.9 ± 1.8
Dark	0.2 ± 0.01	1.3 ± 0.2

$^{14}CO_2$ fixation rates in the dark and light are shown in Table VII. Dark fixation by *Codium* was minimal (0.5% of the light rate), and though it was higher in *Udotea*, it still represented only about 6% of the light rate.

DISCUSSION

Negligible rates of CO_2 evolution in the light and low Γ values, both O_2-insensitive, indicate that *Udotea* essentially lacks photorespiratory CO_2 release. This is verified by the minimal O_2 inhibition of photosynthesis. Thus, *Udotea* differs considerably from *Codium* in terms of gas exchange, and the differences cannot be attributed to variations in dark respiration. The responses for *Udotea* are reminiscent of terrestrial C_4 plants or the low-photorespiration state of freshwater angiosperms (6), while those of *Codium* appear more akin to C_3 systems. It is apparent that a number of marine macroalgae do not exhibit a Warburg effect (4, 6, 19, 22). Taxonomic consistency is obscure, as species with and without O_2 inhibition occur in each division of marine macroalgae (6). The 2 mM DIC in seawater cannot account for the lack of a Warburg effect, by CO_2 outcompeting O_2 at the active site of RuBPCO, because *Codium* was O_2-sensitive at this DIC level. Furthermore, the low Γ value of *Udotea* could not be due to high environmental DIC levels.

For *Chondrus* (Rhodophyta), part of the Warburg effect is due to direct photoreduction of O_2, rather than to photorespiration (7). This could account for the persistent O_2 effect seen in *Codium* at 10 mM DIC, where CO_2 was high enough to outcompete O_2. However, the high and O_2-sensitive CO_2 release rates and Γ values and the reduced O_2 inhibition with increasing DIC point to substantial photorespiration in *Codium* at seawater DIC levels.

The ^{14}C labeling of glycolate, glycine, and serine and the presence of P-glycolate phosphatase and glycolate dehydrogenase were also consistent with an active PCO pathway in *Codium*.

Glycolate dehydrogenase in *Codium*, instead of glycolate oxidase, and the high Γ value negate the suggestion that algal glycolate dehydrogenase and oxidase activities may be correlated with low and high Γ values, respectively (13). For *Udotea*, the low labeling of PCO cycle intermediates, a Warburg effect at very low DIC levels and glycolate dehydrogenase activity (29) suggest it has the potential for limited PCO cycle operation.

Whether a DIC uptake mechanism, similar to that of unicellular organisms, operates in marine macroalgae is unresolved. *Codium* and *Udotea* show lower apparent K_m values for free CO_2 than HCO_3^-, but also seem to use HCO_3^- (6), and the abundance of this ion in seawater may make it the major DIC source. For *Codium*, the increased Warburg effect with EZ at high, but not low pH, suggests that HCO_3^- uptake, followed by its CA-catalyzed equilibration with free CO_2, acts as a limited CO_2 concentrating mechanism to lower, but not eliminate, an otherwise greater Warburg effect. The more pronounced Warburg effect in *Codium* chloroplasts at 10 mM DIC, as compared with thalli at 2 mM DIC, supports the concept of a limited CO_2-concentrating mechanism located outside the chloroplast. An extracellular CA has been reported for *Codium* (9), and from the assays and EZ effects on O_2 inhibition in the current study both chloroplastic and extrachloroplastic CA may be present. There was no evidence from gas exchange, enzymic, or pulse-chase studies for C_4-like photosynthesis in *Codium*.

The situation for *Udotea* is markedly different. The inability of EZ to evoke a Warburg effect at high pH, while still inhibiting photosynthesis, suggests that CA is not a component of the mechanism that overcomes O_2 inhibition, but that it may facilitate HCO_3^- use.

The PEPCK activity in *Udotea*, rather than HCO_3^- use, seems to be a key to its reduced photorespiration. To our knowledge, PEPCK activity equal to that of RuBPCO has not been found before in the Chlorophyta, though it occurs in the Chrysophyta and Phaeophyta (20, 22, 24). The evidence points to PEPCK as the enzyme responsible for β-carboxylation in *Udotea*. Its activity was absolutely dependent upon Mn^{2+}, ADP, and PEP; other carboxylating enzymes, such as pyruvate carboxylase and PEPC, were either not detectable or just barely; and ^{14}C-malate and aspartate were produced in the light. Similar substrate specificity and lack of alternate β-carboxylases have been reported for brown algae in which PEPCK occurs (22, 24). Although PEPCK acts as a decarboxylase in terrestrial C_4-PEPCK species (11), the evidence favors a carboxylating role in some marine algae (6, 20, 22, 24).

It has been suggested that PEPCK functions in brown algae as a light-independent carboxylase, to fix CO_2 at night for incorporation into carbohydrates during the day (22, 24), in a system analogous to CAM photosynthesis. This is not the case in *Udotea*, as dark CO_2 fixation was only 6% of the light rate; also, malate pools were small and did not show CAM-like diel fluctuations (JB Reiskind, and G Bowes, unpublished results). Alternatively, in the unicellular alga *Selenastrum*, under N-resupply conditions the C_4 acids formed by PEPC serve as an anapleurotic source of carbon skeletons for the TCA cycle (12). Although an anapleurotic role for PEPCK in *Udotea* cannot be excluded, it appears unlikely because of the low dark fixation rate. Unlike the situation with *Selenastrum*, the label in the neutral and insoluble fractions increased during the chase, but the label in alanine did not. In anapleurosis the converse would be expected (12).

Our current working hypothesis as to the role of PEPCK in *Udotea* is that it complements RuBPCO, possibly by concentrating CO_2 in a C_4-like photosynthetic process, as occurs with PEPC in the submersed freshwater plant *Hydrilla* (27). The activities of enzymes associated with C_4 acid metabolism and PEP regeneration are consistent with this concept. Though enzyme activities were lower than in terrestrial plants, this appears to be typical of submersed macrophytes and coincides with their low photosynthetic rates (6). Except for NADP-malate dehydrogenase and the two malic enzymes, the activities were equal to or in excess of the photosynthetic rate. C_4 enzymes have been detected in extracts from a few marine algae (6, 22–24), though RuBPCO and the PCR cycle usually predominate (6, 22, 24).

The source of the substrate, PEP, for the PEPCK reaction in *Udotea* requires identification. If it originates from 3-P-glycerate of the PCR cycle, this may imply two carboxylation reactions occurring in series (RuBPCO followed by PEPCK) without an intermediate decarboxylation step. It is not clear how such a system could produce C_4-like gas exchange characteristics. An alternative would be for pyruvate, from the decarboxylation of OAA, to serve as the source, as occurs among terrestrial C_4 species (11). This would require pyruvate Pi dikinase, and other enzymes of PEP regeneration, which are present in *Udotea*.

Further evidence for a modified C_4 system comes from the labeling data. In *Udotea*, malate and aspartate were labeled early, and during the unlabeled chase the label was rapidly lost. In *Codium*, these acids were very slow to label, and did not turnover. The rapid turnover of C_4 acids in *Udotea* suggests they are photosynthetic intermediates; whereas, they appear to be minor end-products in *Codium*. The transient increase in the 3-P-glycerate of *Udotea* near the start of the chase is consistent with label passing from the C_4 acids to sugar-P, but the kinetics are not as well-defined as in a terrestrial C_4 system. HCO_3^- uptake by aquatic organisms partially obscures the labeling patterns (22).

The only enzyme detected in *Udotea* extracts with sufficient activity to act as a decarboxylase is also PEPCK. *Udotea* lacks Kranz anatomy or any obvious cellular differentiation to separate the carboxylation and decarboxylation functions between cells. For PEPCK to carry out both functions and avoid futile recycling of CO_2, it would be necessary for the enzyme to be located in both the cytosol and chloroplast. Decarboxylation would be favored in the chloroplast in the light, because ATP is an effective competitive inhibitor of the carboxylation reaction of PEPCK (32). In the brown macroalga *Laminaria*, PEPCK functions not only as a carboxylase, but as a decarboxylase when the C_4 acid or ATP pools are high (32).

In the absence of Kranz anatomy, in order to raise the CO_2 level at the site of RuBPCO, the concentrating effect must occur at the chloroplast. *Chlorella* (2) and *Chlamydomonas* (25) concentrate CO_2 by a DIC uptake mechanism that may operate at the chloroplast envelope. The hypothesized system in *Udotea* would be somewhat analogous. However, after β-carboxylation, malate or aspartate would be transported across the chloroplast envelope, rather than HCO_3^-, followed by decarboxylation and CO_2 fixation into the PCR cycle. The return of pyruvate, alanine, or even some 3-P-glycerate to the cytosol, as a source of PEP, would complete the cycle. All of the enzymes to perform such a cycle have been identified in *Udotea* extracts.

Additional evidence for the role of PEPCK in photosynthesis comes from a related study using a specific inhibitor of PEPCK, 3-mercaptopicolinic acid. Incubation of the *Udotea* thallus with this compound resulted in: an inhibition of PEPCK activity; a reduced photosynthetic rate; an increased Warburg effect; reduced label in C_4 acids; and increased label in PCO cycle intermediates (JB Reiskind, G Bowes, unpublished data). These data provide substantive evidence that PEPCK and C_4 acid formation are largely responsible for the reduced photorespiration in *Udotea*.

The system in *Udotea* does not appear to be as effective as the DIC accumulation mechanism in unicellular organisms, in that a Warburg effect was seen at the lowest DIC levels. From the labeling of malate and aspartate, which was less than in terrestrial C_4 plants, the *Udotea* system seems more limited in operation.

In freshwater, photosynthesis can raise the dissolved O_2 and can substantially lower the DIC level (30). Thus, for freshwater plants, the advantages of an inducible CO_2-concentrating mechanism, whether based on HCO_3^- uptake or a C_4-like system, are apparent (6). In the marine environment, it is unclear to what extent turbulence dampens deleterious O_2 and DIC fluctuations. Because the DIC level of seawater is insufficient to overcome the Warburg effect, some advantage to a CO_2-concentrating system in marine macroalgae can be postulated. It is apparent that, as with terrestrial angiosperms where a single family may possess species with divergent photosynthetic modes (11), the marine macroalgal divisions also exhibit diversity.

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