Photosynthetic Carbon Metabolism of a Marine Grass

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ABSTRACT

The δ13C value of a tropical marine grass Thalassia testudinum is -9.04%. This value is similar to the δ13C value of terrestrial tropical grasses. The δ13C values of the organic acid fraction, the amino acid fraction, the sugar fraction, malic acid, and glucose are: -11.2%, -13.1%, -10.1%, -11.1%, and -11.5%, respectively. The δ13C values of malic acid and glucose of Thalassia are similar to the δ13C values of these intermediates in sorghum leaves and attest to the presence of the photosynthetic C4-dicarboxylic acid pathway in this marine grass. The inorganic HCO3- for the growth of the grass fluctuates between -6.7 to -2.7% during the day. If CO2 fixation in Thalassia is catalyzed by phosphoenolpyruvate carboxylase (which would result in a -3% fractionation between HCO3- and malic acid), the predicted δ13C value for Thalassia would be -9.7 to -5.7%. This range is close to the observed range of -12.6 to -7.8% for Thalassia and agree with the operation of the C4-dicarboxylic acid pathway in this plant. The early products of the fixation of HCO3- in the leaf sections are malic acid and aspartic acid which are similar to the early products of CO2 fixation in C4 terrestrial plants.

Electron microscopy of the leaves of Thalassia reveal thick walled epidermal cells exceedingly rich in mitochondria and C4-type chloroplasts. The mesophyll cells have many different shapes and surround air lacunae which contain O2 and CO2. The mesophyll cells are highly vacuolated and the parietal cytoplasm contains an occasional chloroplast. This chloroplast contains grana but the lamellar system is not as developed as the system in epidermal chloroplasts. Extensive plasmolysis tissue is present but the xylem elements are reduced in this aquatic grass. The vascular tissue is not surrounded by bundle sheath cells.

This work does not establish the exact relation between structure and function in Thalassia, but it does show the C4-type photosynthetic carbon metabolism in this grass involves epidermal and mesophyll cells and internally produced O2 and CO2 in the air lacunae.

The δ13C values of the primitive photosynthetic bacterium Chromatium correlate with the operation of the Calvin cycle in these microorganisms.

Craig (5), Parker (14), Parker and Calder (15), Smith and Epstein (18), Whelan (23), and Doohan and Newcomb (8) have reported the δ13C values of a marine monocot Thalassia testudinum to be between -7.8 and -12.6‰. These values are indicative of a C4 metabolism. Another C4 characteristic of this plant is its high productivity. Thayer et al. (19) showed the annual production of this grass ranges from 200 to 3000 g C/m2. The annual production of cultivated corn is 412 g C/m2. Jagels (10) and Doohan and Newcomb (8) have shown, however, the anatomy of the leaves of this tropical marine grass differs from most terrestrial C4 grasses and lack bundle sheath cells. The purpose of this paper was to compare the δ13C values of metabolic intermediates, CO2 fixation, and anatomy of this tropical marine grass to terrestrial C4 tropical grasses.

MATERIALS AND METHODS

Materials. Sodium 14C-bicarbonate was purchased from New England Nuclear Corporation.

Plants. Plants of Thalassia testudinum were collected in Redfish Bay, Port Aransas, Texas. The plant consists of an underground rhizome which produces shoots and roots. Most of the leaf samples were taken from the young tissue at the base of the leaf under or adjacent to the leaf sheath. Care was taken to wash off any epiphytic algae on these sections but compared to the older leaf tissue the concentration of epiphytic algae is low or negligible on young tissue under or adjacent to the leaf sheath. The leaf samples to be used for δ13C analyses were dried or placed directly in 95% ethyl alcohol. The leaf samples to be used for 14CO2 fixation and electron microscopy studies were brought to the laboratory in chilled sea H2O.

CO2 Fixation. Leaf sections (1 x 1 cm) were cut from the base of the leaf blades. The leaf sections were submerged in 4.9 ml of sterile sea H2O or de-ionized H2O (pH 5.8) and equilibrated in the light at an intensity of 1.15 x 104 μeinstein/m² sec for 5 to 10 min at 30 C. den Hartog (9) and Sculthorpe (16) have shown the growth optimum of Thalassia is 30 C. Following the equilibration period, 100 μCi of 14C-bicarbonate in 0.1 ml (specific radioactivity 1 mCi/0.142 mmol) was added to the incubation medium. The leaf sections were allowed to fix the radioactive bicarbonate for various lengths of time, and the reactions were terminated with boiling 80% ethyl alcohol.

Extraction of Radioactive Compounds. Following 14CO2 fixation, the leaf sections were extracted 4 times with boiling 80% ethyl alcohol and 4 times with boiling 95% ethyl alcohol. For each extraction, the leaf sections were simmered on the hot plate for 10 to 15 min. The alcohol extracts were pooled, and formic acid was added to liberate the unreacted 14C-bicarbonate. The extract was evaporated to dryness in vacuo. The residue was partitioned between ethyl ether and H2O. An aliquot of the ether phase which contained pigments and lipids was added to a scintillation vial, evaporated to dryness with N2, dissolved in scintillation cocktail, and assayed for radioactivity. The H2O

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2. Abbreviations: RuDP: ribulose-1,5-diphosphate; PEP: phosphoenolpyruvate; PGA: 3-phosphoglycerate; PDB: The 14C/12C ratio in the sample compared to a standard which is CO2 from the fossil carbonate of Belemnella americana.
phase was evaporated to dryness in vacuo. The residue was dissolved in a known volume of H2O, and an aliquot was assayed for radioactivity. The H2O-soluble extract was separated into basic, acidic, and neutral fractions. The H2O-soluble extract was passed through a column (1 × 15 cm) of Dowex-50 (H+)-resin. The basic fraction, consisting of 14C-amino acids, was eluted from the resin column with 50 ml of 1 N NH4OH, and the solution was evaporated to dryness. The effluent from the Dowex-50 column was passed through a column (1 × 15 cm) of Dowex-1 (formate) resin. The acidic fraction, consisting of 14C-organic acids, was eluted from the resin column with 50 ml of 8 N formic acid, and the solution was evaporated to dryness. The effluent from the Dowex-1 column, which consisted of 14C-sugars, was evaporated to dryness. The individual fractions were dissolved in H2O, and an aliquot was assayed for radioactivity.

**Chromatography.** The amino acid, organic acid, and sugar fractions were resolved into individual components by two-dimensional paper chromatography by the procedure of Benson et al. (4). The radioactive compounds were localized on the chromatograms by radioautography on medical x-ray film. The 14C-compounds were cut from the chromatograms and assayed directly for radioactivity by liquid scintillation counting of the filter paper. For identification the radioactive compounds were eluted from the paper chromatograms and co-chromatographed with authentic compounds followed by radioautography.

**30°C Analysis. (500 g)** Leaf sections were repeatedly extracted with boiling 80% and 95% ethyl alcohol. The extracts were pooled and evaporated to dryness. The residue was partitioned between ethyl ether and H2O. The H2O-soluble phase was separated into amino acid, organic acid and sugar fractions by passage through columns (2 × 25 cm) of Dowex resins by the procedure described above. The individual fractions were evaporated to dryness, dissolved in 5 to 10 ml H2O and 0.5 ml applied stripwise to Whatman No. 3 filter papers. The extracts were separated by one-dimensional chromatography in the following solvents: 1, equal volumes of 1-butanol-H2O (370:25 v/v) and propionic acid-H2O (180:220, v/v) for amino acids and organic acids; and 2, ethyl acetate-acetic acid-H2O (150:50:100, v/v) for sugars. Malic acid, glucose, and fructose were analytically separated by this chromatography. These compounds were located and eluted from the paper chromatograms, pooled and evaporated to dryness. The individual compounds were enriched 1.1% in 30 during the isolation procedure.

Carbon isotope ratios were measured on a 60° sector, Nier-type mass spectrometer similar to the one described by Mc Kinney et al. (12). All of the samples were converted to CO2 by combustion at 800 to 900°C over cupric oxide and in an excess O2 atmosphere. The combustion products were circulated continuously by means of an electrically controlled Toepler pump. After removal of H2O vapor and other condensible gases by passing through traps, the samples were cooled to dry ice temperatures and the CO2 was distilled into a sample bulb at liquid N2 temperatures.

**Electron Microscopy.** The leaf sections were fixed in 1.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2. The tissue was then exposed to a phosphate buffered osmium tetroxide fixation, dehydrated with ethanol and embedded in Epon. The sections were stained with uranyl acetate and lead citrate. The sections were photographed with a Hitachi HU-11E electron microscope. The technique for light microscopy involved staining 2.5-μm sections from the Epon-embedded tissue with 1% methylene blue buffered with 1% sodium borate at 80°C.

**RESULTS AND DISCUSSION**

The data in Table 1 show the 613C values of leaf blades and metabolic intermediates of Thalassia. The 613C value of -9.04‰ for the leaf blades compares favorably with the published values of -7.8 to -12.6‰ for similar material (5, 8, 14, 15, 18, 23). These high 613C values are similar to the high 613C values of terrestrial C4 plants (2, 18, 20, 21).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>613C Values</th>
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<tbody>
<tr>
<td>Whole Leaf</td>
<td>-9.04‰</td>
</tr>
<tr>
<td>Organic Acid</td>
<td>-11.2</td>
</tr>
<tr>
<td>Amino Acid Fraction</td>
<td>-13.1</td>
</tr>
<tr>
<td>Sugar Fraction</td>
<td>-10.1</td>
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<tr>
<td>Malic Acid</td>
<td>-11.1</td>
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<tr>
<td>Glucose</td>
<td>-11.5</td>
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<tr>
<td>Fructose</td>
<td>-11.1</td>
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Table 1. 613C Values of Metabolic Intermediates

The data in Table 1 show the 613C values of leaf blades and metabolic intermediates of Thalassia. The 613C value of -9.04‰ for the leaf blades compares favorably with the published values of -7.8 to -12.6‰ for similar material (5, 8, 14, 15, 18, 23).

The 613C values of the organic acid, amino acid, and sugar fractions of the marine grass are -11.2‰, -13.1‰, and -10.1‰, respectively. More specifically, the 613C values of malic acid and glucose are -11.1‰ and -11.5‰. The 613C values of the metabolic intermediates of Thalassia are similar to the 613C values of these intermediates in C4 plants. In sorghum (C4), the 613C values of organic acid, amino acid, and sugar fractions are: -12.3‰, -12.7‰, and -9.9‰ (24). In cotton (C3 plant) the 613C values of these fractions are: -24.8‰, -33.1‰, and -26.4‰ (24). The 613C values of malic acid and glucose from leaves of sorghum and cotton are: -8.9‰, -9.9‰, -21‰, and -24.6‰, respectively.

The pathway of synthesis of sugars from CO2 in terrestrial C4 plants is through malic acid by the Hatch and Slack pathway. The fact that the 613C values of malic acid and glucose in sorghum are similar, reflects the synthesis of glucose from the β-COOH group of malic acid by the C4-dicarboxylic acid pathway. If glucose was synthesized exclusively through the Calvin cycle in sorghum leaves the 613C value of glucose would be about -25‰, as found in C3 plants. The fact that the 613C values of glucose and malic acid in Thalassia are similar to the 613C values of these compounds in sorghum attests to the presence of the photosynthetic C4-dicarboxylic acid pathway in the marine grass.

Parker (14) has shown that the 613C value of the inorganic C from Redfish Bay is -6.7‰. He further showed that the inorganic C of sea H2O in an experimental pond varied 4‰ between 12 noon and 6 PM due to a preferential use of 13C by photosynthesis in the marine plants and algae. Applying this 4‰ variation to the 613C value of inorganic carbon in Redfish Bay, the inorganic C would vary between -6.7 to -2.7‰ between 12 noon and 6 PM. In sea H2O at pH 8.1 to 8.6, 96‰ of the total inorganic carbon is HCO3- (14). The 613C value of dissolved CO2 during this time would be -13.5 to -9.5‰ (a correction of -6.8‰ is applied to C isotope fractionation between gaseous CO2 and dissolved HCO3- at 30°C, ref. 7).

If all of the CO2 fixation by Thalassia is catalyzed by RuDP carboxylase (which would result in a 18.3‰ fractionation between CO2 and PGA, 24) the 613C value of Thalassia would be between -31.8 to -27.8‰. On the other hand, if the CO2 fixation of Thalassia is catalyzed by PEP carboxylase (which would result in a -3‰ fractionation between HCO3- and malic acid, ref. 24) the 613C value of Thalassia would be -9.7 to -5.7‰. This latter range is close to the observed 613C values of Thalassia (4, 14, 15, 18, 23) and is in agreement with the operation of a C4-dicarboxylic acid pathway in this plant.

The results in Figure 1 show the relative per cent of the total 14C-bicarbonate which is incorporated into amino acids, organic acids, and sugars. After 5 sec of incubation, 55‰ of the label is in the organic acids, 39‰ in amino acids, and 0‰ in the sugars. In 10 min there is a net synthesis of sugars. Chromatography and radioautography of the reaction products indicated that malic acid and aspartic acids were the earliest stable products of CO2 fixation. After 5 sec of exposure of the leaf sections to H14CO3-.
The reduced vascular tissue is located between the epidermal mechanism of osmoregulation. The leaves have no cuticle or stomates. The mesophyll cells are undifferentiated and the reduced vascular tissue is located between the fiber cells. There are no bundle sheath cells around the vascular tissue. The epidermal cells are rich in chloroplasts and mitochondria, and contain highly invaginated plasmalemma which functions in salt secretion. No particular emphasis was placed on the type of chloroplast in the epidermal cells and no chloroplasts were demonstrated in the mesophyll cells. We have studied the ultrastructure of *Thalassia* aiming toward a fuller description of the photosynthetic characteristics of the epidermal and mesophyll cells. This is important because (a) the δ13C values and early products of CO₂ fixation indicate the presence of a C₄-dicarboxylic acid pathway in this monocot, and (b) C₄ metabolism in terrestrial grasses is tightly associated with the mesophyll and bundle sheath cells of the leaves.

A cross section of a leaf of *Thalassia* shows the epidermal cells contain chloroplasts and enclose mesophyll cells and air lacunae. Figure 3 shows the cross sectional relation between the epidermis, mesophyll, and air lacunae. The outer epidermal wall is thickened and the mesophyll cells have different morphological shapes. Different size air lacunae are present. The mesophyll cells surround the air lacunae. It has been shown (26) that the air lacunae contain O₂ and CO₂.

The ultrastructure of an epidermal cell of *Thalassia* is shown in Figure 4A. The cell is packed with chloroplasts. Higher magnifications show many prominent mitochondria in the darker staining layer of cytoplasm. The cell contains a prominent cell wall with striations or layers. The outer epidermal wall is much thicker than the walls between adjacent epidermal cells or between epidermal and mesophyll cells. The cytoplasm and chloroplasts contain abundant osmophillic granules and droplets. The epidermal cells of *Thalassia* differ from the epidermal cells of terrestrial C₄ grasses which do not contain chloroplasts. Figure 4B shows a higher magnification of an epidermal chloroplast. The chloroplast contains a well developed grana system and resembles the C₄ type of chloroplast in the mesophyll cells of C₄ plants. There are osmophillic droplets in the chloroplasts but the chloroplasts do not contain starch granules. This electron photomicrograph shows striations or layers in the cell wall. Albersheim (1) has concluded these repeating structures have the expected dimensions of the primary wall in plants. Figure 4C is an electron photomicrograph of a mesophyll cell surrounding an air lacunae. The mesophyll cell is highly vacuolated and the parietal layer of cytoplasm contains an occasional chloroplast. Figure 4D shows a higher magnification of a mesophyll chloroplast. The chloroplast does contain grana but the grana and stroma lamellae do not fill the chloroplast. The lamellar system in these chloroplasts is not as well developed as the system in epidermal chloroplasts.

![Image](https://example.com/image.png)

**Fig. 1.** Relative per cent of total radioactivity incorporated into leaf sections. The rate of ¹⁴CO₂ incorporation into the leaf sections was 1.008 mg C/g dry wt·hr which compares with CO₂ fixation in Zostera (13) of 1.18 mg C/g dry wt·hr.

![Image](https://example.com/image.png)

**Fig. 2.** Relative per cent of total radioactivity incorporated into metabolic intermediates. Following the 5-min incubation period approximately 12% of the total radioactivity was recovered in unknown organic acids and 12% was recovered in unknown amino acids.

33% of the label is in malic acid, 30% in aspartic acid, and less than 2% in PGA. The results in Figure 2 show the relative per cent of the total radioactivity which is incorporated into C₄ acids (malic acid and aspartic acid), PGA, glutamic acid, citric acid, and hexose-phosphates. PGA is only slowly labeled in 1 min of incubation and then shows a negative slope to 10 min of incubation. The C₄ acids show strikingly negative slopes of isotope incorporation which indicates one site of CO₂ incorporation. The first labeled products of CO₂ fixation are malic acid and aspartic acid which is similar to the early products of CO₂ fixation in C₄ plants.

Figure 4A shows a cross sectional view of epidermis, mesophyll, and air lacunae in a leaf of *Thalassia*. E: epidermal cell; M: mesophyll cell; AL: air lacunae. × 160.

Figure 5 is an electron photomicrograph of the vascular tissue between the fiber cells and epidermal cells of *Thalassia*. The vascular tissue is not surrounded by bundle sheath cells. The phloem tissue is extensive but like many submerged aquatic plants the xylem tissue is greatly reduced (16). Highly vacuolated mesophyll cells are present between phloem cells and fiber cells. There does not seem to be any anatomical arrangement of vascular bundles and bundle sheath cells of the Kranz anatomy of terrestrial C₄ plants.

In conclusion, the δ¹³C analysis of the metabolic intermediates and CO₂ fixation studies show that the photosynthetic carbon metabolism of *Thalassia* is similar to C₄-type metabolism. Yet the electron microscopy studies show the anatomy of the tropical marine grass is decidedly different than tropical C₄ grasses. It is important to note that Shomer-Ilan *et al.* (17) have shown that *Suaeda monoica* lacks bundle sheath cells but is a C₄ plant. The C₄ metabolism in these succulent leaves probably involves two types of chlorenchymatic cells. This work does not establish the relationship between structure and function in *Thalassia* but many features are noteworthy. The epidermal cells appear to have active photosynthetic and mitochondrial metabolism associated with osmoregulation (10). These cells may be the primary
CO₂ fixation site and CO₂ uptake may be associated with osmo-regulation. The CO₂ fixation products in the epidermal cells may be transported to the mesophyll cells but Jagels (10) was unable to detect plasmodesmata between these cells. The mesophyll cells contain a small population of chloroplasts and probably could directly fix CO₂. The leaves contain a well developed system of air lacunae surrounded by mesophyll cells. The internal gas (CO₂ and O₂) production and storage in the air lacunae swells the leaf blades 200 to 250% during the day (26). The mesophyll cells could probably re-fix any respired CO₂ in these lacunae. Thalassia lacks bundle sheath cells but photosynthetic carbon metabolism in the epidermal and mesophyll cells together with the recycling of CO₂ in the air lacunae may be a system to account for the high productivity of this tropical marine grass.

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LITERATURE CITED