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Carbon-Isotope Composition of Biochemical Fractions and the Regulation of Carbon Balance in Leaves of the C_3-Crassulacean Acid Metabolism Intermediate *Clusia minor* L. Growing in Trinidad[1]

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Carbon-isotope ratios (δ^{13}Cs) were measured for various biochemical fractions quantitatively extracted from naturally exposed and shaded leaves of the C_3-Crassulacean acid metabolism (CAM) intermediate *Clusia minor*, sampled at dawn and dusk on days during the wet and dry seasons in Trinidad. As the activity of CAM increased in response to decreased availability of water and higher photon flux density, organic acids and soluble sugars were enriched in ^{13}C by approximately 3.5 to 4‰ compared to plants sampled during the wet season. The induction of CAM was accompanied by a doubling in size of the reserve carbohydrate pools. Moreover, stoichiometric measurements indicated that degradation of both chloroplastic reserves and soluble sugars were necessary to supply phospho*enol*pyruvate for the synthesis of organic acids at night. Results also suggest that two pools of soluble sugars exist in leaves of *C. minor* that perform CAM, one a vacuolar pool enriched in ^{13}C and the second a transport pool depleted in ^{13}C. Estimates of carbon-isotope discrimination expressed during CAM, derived from the trafficking among inorganic carbon, organic acids, and carbohydrate pools overnight, ranged from 0.9 to 3.1‰. The δ^{13}C of structural material did not change significantly between wet and dry seasons, indicating that most of the carbon used in growth was derived from C_3 carboxylation.

Clusia minor L. is a tropical dicotyledonous tree that shows extreme flexibility in regulating the relative amounts of CO_2 fixed during the day and night in response to changes in the environment (Franco et al., 1990, 1992; Borland et al., 1992, 1993; Winter et al., 1992; Zotz and Winter, 1993). Under natural conditions, short-term changes in on-line, instantaneous Δ have illustrated how shifts in C_3 and C_4 carboxylation occur on a daily basis in *C. minor*, reflecting changes in environmental parameters (Borland et al., 1993). These variations in carboxylation pathway may in the first instance maximize net carbon uptake and maintain photochemical integrity (Borland and Griffiths, 1994). In the longer term, the shift toward increased CAM activity in response to a diminishing water supply extends the period of carbon gain into the dry season when flowering and seedset occurs in *C. minor*.

Despite the considerable capacity for CAM in *C. minor* (Borland et al., 1992) and the observation that C_4 carboxylation can account for up to 30% of CO_2 fixed during the day in the dry season (Borland et al., 1993), δ^{13}Cs of leaf organic material reveal that during the annual cycle of leaf growth most carbon is fixed via the C_3 pathway (Borland et al., 1992). However, given the large diel changes in the size of organic acid and carbohydrate pools in CAM plants, measurements of δ^{13}C of different biochemical fractions at dawn and dusk could provide a means of reconciling hourly changes in instantaneous Δ with seasonal changes in δ^{13}C. Analysis of carbohydrate pool δ^{13}C has also been used to infer pathways of glycolysis operating at night during glucan mobilization for CAM in *Bryophyllum daigremontianum* (Deléens and Garnier-Dardart, 1977). In contrast to many CAM plants, in *Clusia* sp. soluble sugars form the major reserve carbohydrate pool, and both malic and citric acids may be accumulated at night (Popp et al., 1988; Borland et al., 1992; Franco et al., 1992). Moreover, it has recently been suggested that partitioning of fixed carbon between various biochemical fractions during the day controls the rapid switches between C_3 and CAM in *Clusia uvitana* (Winter et al., 1992; Zotz and Winter, 1993). The level of leaf-sap titratable acidity at dusk is thought to "sense" the requirement for nocturnal CO_2 uptake (Winter et al., 1992; Zotz and Winter, 1993), specifically through the concentration of citric acid (A.M. Borland, unpublished observations).

In the present work the arrangement of carbon pools in naturally shaded and exposed plants of *C. minor*, with both C_3 and CAM characteristics, was assessed via quantitative measurements of the carbon-isotope composition of various organic fractions. To reflect the different sources of CO_2 that can enter various metabolic pools (i.e. external versus respi-

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Abbreviations: δ^{13}C, carbon-isotope ratio; Δ, carbon-isotope discrimination; PFD, photon flux density; PEPC, phospho*enol*pyruvate carboxylase.

ratory CO_2), the $\delta^{13}C$ nomenclature was preferred to Δ nomenclature, which is independent of source CO_2 (Farquhar et al., 1989). Analyses of carbohydrates has been used to provide an integrated measure of the carbon-isotope discrimination expressed on a daily basis in C_3 plants (Brugnoli et al., 1988). Using $\delta^{13}C$ analyses and mass balance interconversions of organic acid and carbohydrate fractions associated with CAM, one can now calculate the carbon-isotope discrimination expressed during the dark period. Leaves were sampled during the wet and dry seasons in Trinidad at dawn and dusk on the days when on-line measurements of instantaneous discrimination had been made (Borland et al., 1993). Analyses of the $\delta^{13}C$ composition of individual metabolic components during the day-night cycle were made to illustrate how products of C_3 and C_4 carboxylation regulate plant carbon balance during daily and seasonal time scales.

MATERIALS AND METHODS

Habitat and Plant Material

Leaves were sampled from plants of *Clusia minor* L. that grew on a site approximately 500 m from the Simla Research Station (10°41'N, 61°17'W; grid reference PS869 823) in the Arima Valley on the island of Trinidad, West Indies. An annual dry season usually extends from late February to April each year with annual rainfall of approximately 2500 mm at Simla. Leaves were sampled at the beginning of February (wet season) and in mid-March (dry season). The exposed population of *C. minor* grew terrestrially on a rocky limestone outcrop with plants reaching a height of 3 to 4 m. The shaded population of plants was found nearby, growing terrestrially beneath a canopy of deciduous seasonal forest. All measurements were conducted on the third leaf pair from the growing tip. These leaves were fully expanded and showed no visible signs of senescence.

Daily incident PFD was monitored from the 3rd week in January until the end of March 1992 using an integrating quantum sensor (Delta T Devices Ltd., Burwell, Cambridge, UK). Daily rainfall was also monitored during this period. All measurements were made on leaves sampled on February 4, 1992 (wet season), and on March 16, 1992 (dry season). The daily PFD was averaged over the week that preceded each of these sampling dates.

Gas Exchange and Respiratory Recycling

The net uptake of CO_2 was measured using a portable IRGA (LCA2; Analytical Development Co. Ltd., Hoddesdon, Hertsfordshire, UK) with an air supply unit drawing air through Teflon tubing from a 25-L mixing volume placed approximately 4 m above the ground with the air intake at the top of the surrounding canopy. A whole leaf was enclosed within the leaf chamber (PLC-3C; ADC) and hourly measurements were made on five replicates each from exposed and shaded leaves. The amount of CO_2 fixed was subsequently integrated over the day and night. Recycling of respiratory CO_2 was calculated as the difference between dawn and dusk changes in malic acid (Δ_{d-d}mal in mmol m^{-2}) and integrated nighttime CO_2 uptake ($\Delta_{d-d}CO_2$ in mmol m^{-2}) assuming that 1 mol of malic acid is synthesized for every 1 mol of CO_2 fixed. This also assumes that a negligible amount of malate was respired directly at night to provide additional energy requirements for H^+ accumulation (Kalt et al., 1990). Citric acid was not included in the calculations of recycling since the accumulation of citric acid at night is unlikely to result in net acquisition of CO_2 (Lüttge, 1988; Haag-Kerwer et al., 1992). Thus, recycling was calculated as:

$$\Delta_{d-d}\text{mal} - \Delta_{d-d}CO_2$$

Extraction and Separation of Biochemical Fractions

Leaves from exposed and shaded plants were sampled at dawn and dusk. Four replicates, each consisting of two leaves, were taken for each regime. The mid-ribs were removed before the leaves were powdered in liquid nitrogen and then placed in 15 cm^3 of 80% methanol and heated at 80°C for 30 min. Extracts were then centrifuged, and the supernatant was decanted. The methanol extraction was repeated until the plant debris was colorless. Chloroform was added to the combined methanol extracts to extract pigments and lipids. The chloroform extract was dried, weighed, and kept for $\delta^{13}C$ analysis. An aliquot of the methanol extract was analyzed for soluble carbohydrates using the phenol-sulfuric acid test (Dubois et al., 1956). The remaining methanol extract was dried, taken up in 1 cm^3 of nanopure water, and applied to columns of ion-exchange Sephadex; SP-C-25-H^+ and QAE-A-25-formate in series. The neutral sugars were washed through with 25 cm^3 of nanopure water before the columns were disconnected and amino acids were washed from the SP-Sephadex with 30 cm^3 of 0.2 M NH_4OH and organic acids from the QAE-Sephadex with 30 cm^3 of 4% formic acid. All the extracts were dried down, weighed and kept for $\delta^{13}C$ analysis. The remaining solid extract was extracted twice with nanopure water at 40°C to remove any oligosaccharides. An aliquot was kept for carbohydrate analysis. The solid residue was washed once with distilled water before 2 × 10 cm^3 of 20% HCl were added for 1.5 h to hydrolyze the remaining carbohydrates, i.e. glucans and starch (Brugnoli et al., 1988). An aliquot of this acid extract was kept for analysis of carbohydrates (i.e. glucans plus starch), and the remainder was made up to 80% ethanol (v/v). These extracts were refrigerated overnight to precipitate the starch, and following centrifugation, the supernatant was decanted and an aliquot was taken for the analysis of carbohydrates (glucans only). The supernatant (glucans) and pellet (starch) were dried and kept for $\delta^{13}C$ analysis. For quantitative purposes and the construction of carbon budgets, starch and glucans were considered as one pool because of the difficulties associated with accurate determination of the small amounts of starch present in the tissue. The remaining solid material was washed thoroughly with distilled water and dried overnight in an oven at 65°C and kept for analysis of $\delta^{13}C$.

There was no carbon-isotope fractionation introduced by the extraction and purification procedures described above. This was tested by analysis of the $\delta^{13}C$ and percentage recovery of analytical grade Suc, malate, citrate, Pro, and starch before and after various stages in the extraction procedure. The concentrations of malate and citrate in the organic acid extract were determined enzymatically according to the methods of Hohorst (1965) and Möllering (1985).

Analysis of δ^{13}Cs

Approximately 4 mg dry weight of each of the organic fractions were sealed in evacuated silica quartz tubes containing CuO and then combusted at 800°C for 6 h. The liberated CO_2 was then purified by cryodistillation through oxidation and reduction columns prior to analysis with an isotope ratio mass spectrometer (Isospec 44; modified by Provac, Crewe, UK) with δ^{13}C expressed with reference to Pee Dee Belemnite standard.

RESULTS

Expression of CAM in the Wet and Dry Seasons

Shaded leaves demonstrated C_3 characteristics in the wet season, whereas exposed leaves demonstrated CAM cycling with malic acid formed from the refixation of respiratory CO_2 at night (Fig. 1a; Table I). Gross respiratory rates, calculated as the sum of recycling plus net efflux of CO_2 at night, were equivalent to 12.5 mmol CO_2 m^{-2} $night^{-1}$ (or 0.29 μmol m^{-2} s^{-1}) for exposed leaves and 6.2 mmol m^{-2} $night^{-1}$ (0.14 μmol m^{-2} s^{-1}) for shaded leaves. CAM activity had been fully induced approximately 6 weeks later in the dry season, with dark uptake of CO_2 (net uptake plus recycling) accounting for 35% (exposed) and 52% (shaded) of the carbon fixed during 24 h, with shaded leaves recycling a higher proportion of respiratory CO_2 (Table I). Moreover, the higher incident PFD, typically encountered during the dry season, was accompanied by a substantial increase in total CO_2 uptake over 24 h, compared to plants sampled during the wet season.

During the wet season, citric acid was accumulated at night in preference to malic acid in the CAM-cycling exposed leaves, whereas the shaded leaves maintained a high background concentration throughout the day-night cycle (Fig. 1). Since CAM was enhanced in the dry season, dawn and dusk concentrations of citric acid declined with malic acid predominantly accumulated at night. Carbohydrate sources for the provision of PEP at night consisted of soluble sugars and, to a lesser extent, glucans and starch, with pools increased at dusk in proportion to the extent of CAM activity (Fig. 2). In exposed leaves, soluble sugars provided twice as many hexose units compared to the glucan pool, which included starch. In shaded leaves similar proportions of soluble sugars and glucans were degraded overnight (Table II). Degradation of both soluble sugars and glucans was necessary to supply the carbon skeletons for organic acid synthesis. The remaining hexose units were partitioned between respiration and export, assuming that rates of gross dark respiration calculated from the data in Table I represent CO_2 generated from hexose via the Krebs cycle. Leaves with C_3 characteristics (shaded, wet season) exported most carbohydrates (i.e. Suc) at night, with CAM plants exporting substantially less, although a positive carbon balance was still maintained at night.

δ^{13}Cs of Biochemical Fractions

Wet Season

Approximately half of the leaf dry weight was composed of structural material (Fig. 3, a and b) with the δ^{13}C of this fraction similar to that of the whole leaf (Table III). Exposed leaves were relatively enriched in ^{13}C, with δ^{13}C of all organic fractions approximately 4‰ less negative than shaded leaves. At dusk, carbohydrates constituted approximately 30% of dry weight in exposed leaves (CAM cycling) but only 15% in shaded leaves (C_3; Fig. 3, a and b). Conversely, the organic acid fraction constituted 17% of dry weight in shaded C_3 leaves, compared to only 6% in leaves with CAM cycling. The δ^{13}C of these fractions remained constant throughout the day-night cycle in shaded plants (Table III). However, products of CAM cycling enriched in ^{13}C were transferred from organic acid to soluble sugar pool, as shown by a reciprocal shift of 2‰ between these fractions in exposed levels (Table III).

Dry Season

Following the induction of CAM activity in the dry season, the δ^{13}Cs of whole leaves, structural material, starch, and amino acid fractions were similar to those in the wet season (Tables III and IV). The carbon-isotope composition reflected the increased day-night changes in organic acid and carbohydrate pools (Figs. 1 and 2). The δ^{13}C of values of organic acids at dawn were 3 to 5‰ less negative in exposed and shaded leaves, respectively, compared to those isolated dur-

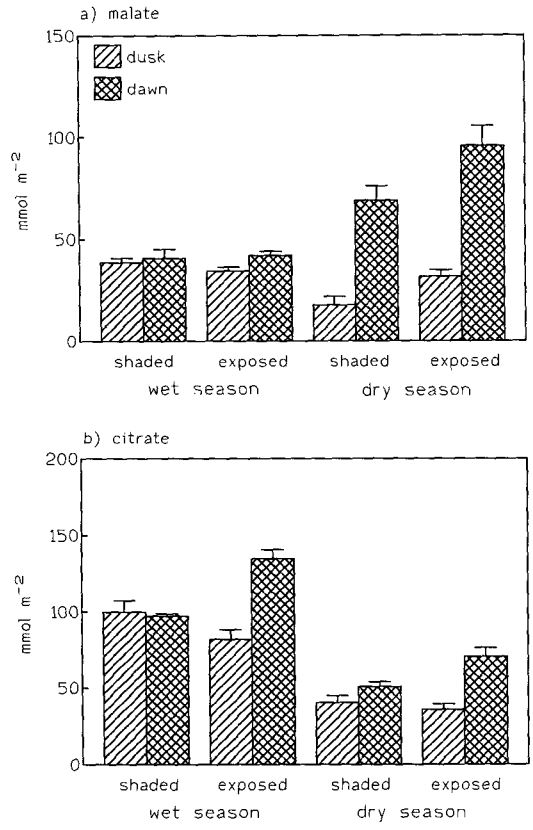

Figure 1. Concentrations of malate (a) and citrate (b) in exposed and shaded leaves of *C. minor* sampled at dawn and dusk on days during the wet and dry seasons in Trinidad. Data are the means of four replicates ± SE.

Table I. *Photosynthetic characteristics of C. minor*

Integrated net CO_2 uptake during day and night for 24-h periods during the wet and dry seasons in Trinidad, calculated from hourly measurements on five replicate leaves each from exposed and shaded plants. SE values <10%. The amount of respiratory CO_2 fixed for the synthesis of malate at night is also shown together with total rainfall and average daily PFD for the week preceding the date of sampling and data for leaf succulence, where $n = 8$.

Habitat	Rainfall	Daily PFD	Integrated Net CO_2 Uptake		Respiratory Recycling	Succulence
			Day	Night		
	mm week^{-1}	*mol* m^{-2}	*mmol* m^{-2}		*mmol* m^{-2}	*g* m^{-2}
Wet season exposed	11.2	29.4 ± 1.8	73.9	−6.2	6.3	625.9 ± 8.7
Shaded	11.2	5.2 ± 0.2	62.1	−6.2	0	519.1 ± 8.6
Dry season exposed	0	39.6 ± 1.4	120.4	52.0	10	565.4 ± 10.6
Shaded	0	9.9 ± 0.3	52.6	29.6	21.0	480.0 ± 8.5

ing the wet season, showing the major contribution from nocturnal PEPC carboxylation. Both soluble sugar and glucan pools became enriched in ^{13}C during the course of the day as the "C_4" label was transferred from the organic acid pool (Table IV).

Carbon-Isotope Composition of Mobile Carbon Pools

If one knows the absolute change in concentration and $\delta^{13}C$ of organic acid and carbohydrate pools overnight, it is possible to calculate the isotopic signature of each "mobile" carbon pool using the mass balance equation developed by Deléens and Garnier-Dardart, (1977):

$$\delta^{13}C = \frac{(\delta^{13}C_m \times M_m) - (\delta^{13}C_e \times M_e)}{(M_m - M_e)}$$

where $\delta^{13}C$ = isotopic signature of carbon lost or acquired by a particular metabolic pool, M_m = mass of product (% dry weight) with $\delta^{13}C_m$ value (dawn), and M_e = mass of product (% dry weight) with $\delta^{13}C_e$ value (dusk).

For example, the isotopic signature of carbon lost overnight from the soluble sugar pool in exposed leaves in the dry

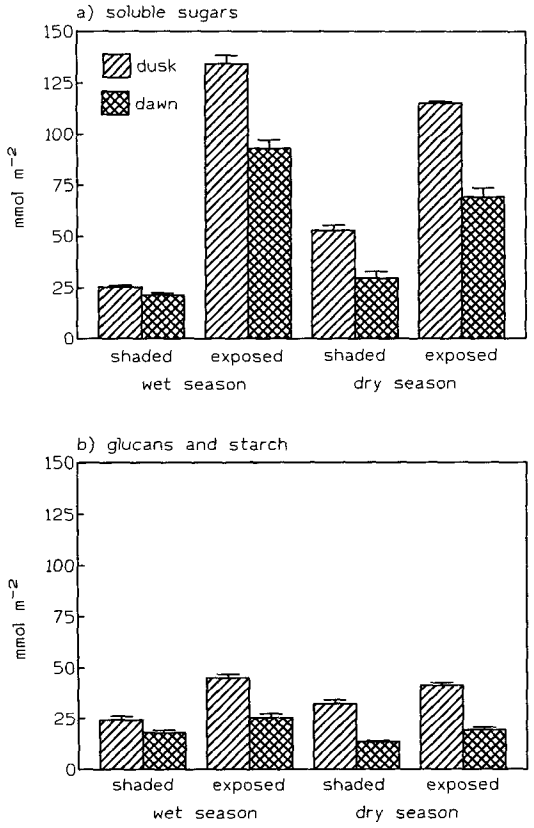

Figure 2. Concentrations of soluble sugars (a) and glucans plus starch (b) in exposed and shaded leaves of C. *minor* sampled at dawn and dusk on days during the wet and dry seasons in Trinidad. Data are the means of four replicates ± SE.

Table II. *Stoichiometry of carbohydrate depletion and organic acid accumulation at night*

Dawn-dusk changes (Δ_{d-d}) in carbohydrate and organic acid pools were measured for exposed and shaded leaves of *C. minor* sampled during the wet and dry seasons in Trinidad. It was assumed that 1 mol of hexose could be used for the synthesis of 2 mols of malate or 1 mol of citrate. Hexose units surplus to requirements for organic acid synthesis were assumed to supply all respiratory CO_2 at night (see Table I) with 1 mol of hexose generating 6 mol of CO_2. The remaining hexose units were assumed as export at night.

	Wet Season		Dry Season	
	Exposed	Shaded	Exposed	Shaded
Δ_{d-d} soluble sugar (mmol m^{-2})	−40.8	−3.7	−46.0	−23.4
Δ_{d-d} glucans (mmol m^{-2})	−19.7	−6.1	−21.9	−18.7
Δ_{d-d} total hexose (mmol m^{-2})	−60.5	−9.8	−67.9	−42.1
Δ_{d-d} malate (mmol m^{-2})	+6.2	0	+62.0	+50.6
Δ_{d-d} citrate (mmol m^{-2})	+53.0	0	+34.7	+10.4
Δ_{d-d} hexose equivalents (mmol m^{-2})	+56.1	0	+65.7	+35.7
Respired hexose (mmol m^{-2})	2.1	1.0	1.7	3.5
Exported hexose (mmol m^{-2})	2.3	8.8	0.5	2.9

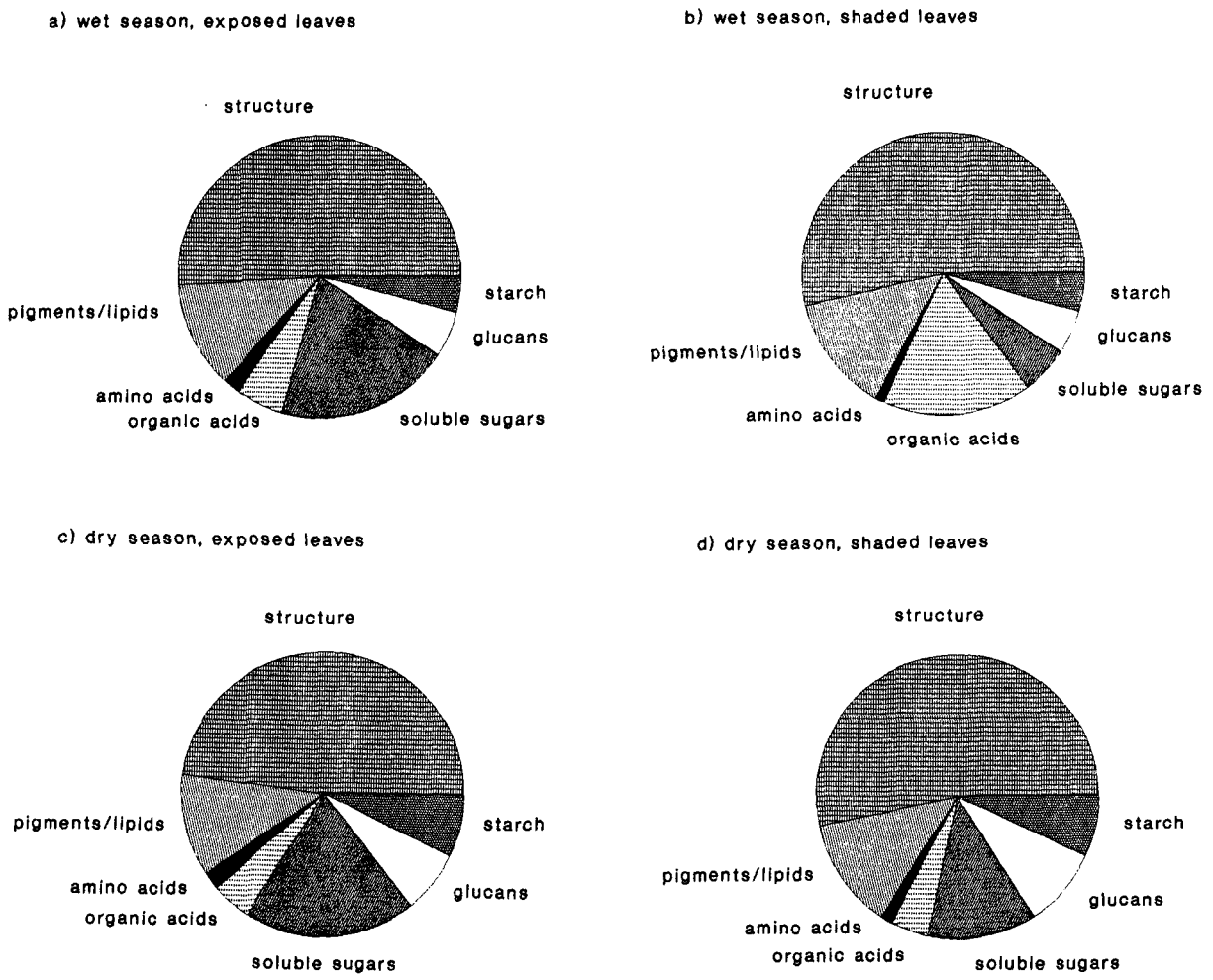

Figure 3. The relative dry weight proportions of various fractions extracted from exposed (a and c) and shaded (b and d) leaves of *C. minor* sampled at dusk on days during the wet (a and b) and dry (c and d) seasons in Trinidad.

season was:

$$\frac{(-20.4 \times 12) - (-17.9 \times 19.7)}{(12 - 19.7)} = -14.0‰$$

Therefore, we may derive the $\delta^{13}C$ of the carbon fixed into organic acids overnight and compare this with the change in $\delta^{13}C$ of the carbohydrate pool that has provided the residual carbon skeleton (Table V). Carbon lost from soluble sugar and glucan pools, both serving as substrates for organic acid synthesis (Fig. 2; Table II), was more enriched in ^{13}C than CO_2 fixed during phase IV in leaves with CAM activity (Table V). Thus, the carbon skeletons utilized from soluble sugars and glucans had a less negative (more CAM-like) $\delta^{13}C$ value, whereas the starch used was more likely to have been derived from phase IV C_3 carboxylation, since the isotope signature was similar to the instantaneous discrimination expressed at this time (Table V, using data derived from Borland et al., 1993).

Having determined the relative proportion of the different carbohydrate pools that contribute carbon skeletons used in organic acid synthesis (Table II), one may express the pools as a percentage of the total carbon units available. The percentage of carbon fixed from atmospheric CO_2 has been calculated from Table I, with the $\delta^{13}C$ having been measured directly (Table V; Borland et al., 1993). Knowing the $\delta^{13}C$ of source and product allows one to derive the difference in average carbon-isotope composition between carbohydrate pool and organic acid for each regime. In effect, such a value represents the carbon-isotope discrimination that had been expressed during carboxylation by PEPC. Thus, calculated discrimination ranged from 0.9 to 3.1‰ (exposed and shaded plants, respectively, in the dry season; Table V), in parallel with the magnitude of CAM induction (Figs. 1 and 2).

DISCUSSION

Although CAM is usually considered to represent the temporal separation of C_3 and C_4 carboxylation, it is clear that during the day carbohydrate reserves intended for CAM must be marshalled independently from carbon skeletons destined for nitrogen metabolism, respiration, and growth. Studies on the facultative *Kalanchoë blossfeldiana* and constitutive *B. daigremontianum* suggested compartmental partitioning of

Table III. $\delta^{13}C$ (‰) *of biochemical fractions quantitatively isolated during the wet season*

Naturally exposed and shaded leaves of *C. minor* were collected at dawn and dusk on February 4, 1992, and data are the means of four replicates ± SE.

Fraction	Exposed Leaves[a]		Shaded Leaves[a]	
	Dawn	Dusk	Dawn	Dusk
Pigments and lipids	−28.2 ± 0.2	−28.7 ± 0.2	−33.3 ± 0.7	−32.2 ± 0.5
Amino acids	−30.2 ± 0.6	−31.4 ± 0.4	−32.0 ± 1.0	−32.6 ± 0.5
Organic acids	−20.6 ± 0.5	−22.3 ± 0.6	−27.6 ± 0.2	−27.7 ± 0.5
Soluble sugars	−23.6 ± 0.8	−21.2 ± 0.3	−30.5 ± 0.2	−29.2 ± 0.2
Glucans	−22.6 ± 0.4	−22.3 ± 0.6	−27.3 ± 0.2	−27.6 ± 0.6
Starch	−22.1 ± 0.3	−22.3 ± 0.3	−27.2 ± 0.4	−27.8 ± 0.9
Structure	−26.1 ± 0.9	−27.2 ± 0.2	−31.2 ± 0.4	−31.4 ± 0.6

[a] Whole leaf: exposed, $\delta^{13}C = -25.7 \pm 1.9$‰; shaded, $\delta^{13}C = -30.3 \pm 2.3$‰.

carbon fluxes between C_3 and C_4 metabolism in plants performing CAM. Thus, at night, respiratory CO_2 was generated from soluble sugars depleted in ^{13}C, whereas PEP was produced from the glycolytic breakdown of ^{13}C-enriched starch (Deléens and Garnier-Dardart, 1977; Deléens et al., 1979). In *C. minor*, however, the situation appears more complex with degradation of both soluble sugars and starch supplying PEP for the synthesis of malate and citrate (Table II; Popp et al., 1988).

Energetics of Carbon Fluxes in *C. minor*

The costs associated with the dark reactions of CAM depend on the nature of the carbohydrate source of PEP and the relative proportions of malic and citric acids accumulated. With starch as the hexose donor, 0.5 hexose plus $CO_2 \rightarrow 1$ mal plus 0.5 ATP. Since the accumulation of malic acid in the vacuole requires 1 ATP to drive the tonoplast ATPase, there is an overall energy requirement of 0.5 ATP per malate. Conversely, the synthesis of citrate may potentially result in the production of 9 ATP, 3 in the cytosol and 6 in the mitochondrion (Lüttge, 1988). Since transport of pyruvate into the mitochondrion may impose a cost of 1 ATP and accumulation of citric acid in the vacuole requires 1.5 ATP, the net stoichiometry is 1 hexose → 1 citric acid plus 6.5 ATP. Alternatively, when soluble sugars serve as the source of PEP, the ATP requirements of the reaction catalyzed by hexokinase means that accumulation of malic acid requires 1 ATP, whereas citric acid accumulation will potentially generate 5.5 ATP. However, it has been suggested that PPi:Fru-6-P 1-phosphotransferase, which uses PPi to phosphorylate Fru-6-P → Fru-1,6-P_2, rather than the ATP-requiring phosphofructokinase, may catalyze the first irreversible step in glycolytic production of PEP in CAM plants that store soluble sugars (Fahrendorf et al., 1987; Carnal and Black, 1989). This would result in similar ATP requirements for malic acid accumulation and ATP production from citric acid accumulation regardless of whether starch or soluble sugars served as the source of PEP. Energetics of the different routes of carbohydrate breakdown are calculated in Table VI and compared with the amount of ATP potentially available from dark respiration and citric acid accumulation. In theory, respiratory activity was sufficient to supply all of the ATP required for malic acid accumulation by either pathway, with citric acid accumulation potentially generating supplementary ATP.

Carbon Trafficking and Carbon-Isotope Composition during the Dark Period

The synthesis of citrate from carbohydrate reserves does not result in net acquisition of CO_2 (Lüttge, 1988; Fig. 4a). However, in exposed plants of *C. minor* sampled in the wet season, the organic acids accumulated overnight (90% of

Table IV. $\delta^{13}C$ (‰) *of biochemical fractions quantitatively isolated during the dry season*

Naturally exposed and shaded leaves of *C. minor* were collected at dawn and dusk on March 16, 1992, and data are the means of four replicates ± SE.

Fraction	Exposed Leaves[a]		Shaded Leaves[a]	
	Dawn	Dusk	Dawn	Dusk
Pigments and lipids	−27.1 ± 1.2	−27.7 ± 1.2	−30.8 ± 0.8	−30.8 ± 0.3
Amino acids	−31.2 ± 0.6	−31.1 ± 0.9	−32.4 ± 1.4	−32.7 ± 1.3
Organic acids	−17.6 ± 0.7	−21.1 ± 0.6	−21.8 ± 0.8	−24.5 ± 0.6
Soluble sugars	−20.4 ± 0.9	−17.9 ± 0.4	−24.3 ± 0.7	−21.4 ± 0.4
Glucans	−23.4 ± 0.4	−21.6 ± 0.4	−26.8 ± 0.7	−23.6 ± 0.6
Starch	−23.6 ± 0.3	−23.1 ± 0.5	−27.9 ± 0.2	−26.6 ± 0.3
Structure	−27.1 ± 1.4	−27.5 ± 0.5	−31.8 ± 0.4	−31.0 ± 0.4

[a] Whole leaf: exposed, $\delta^{13}C = -24.6 \pm 1.1$‰; shaded, $\delta^{13}C = -29.1 \pm 1.8$‰.

Table V. *$\delta^{13}C$ (‰) of mobile pools of organic acids and carbohydrates at night*

The $\delta^{13}C$ of CO_2 fixed during phase IV was reworked from data by Borland et al. (1993) and the percentage of carbon derived from the carbohydrate pools and from the atmosphere that was required for the synthesis of organic acids is shown in parentheses. The difference between the $\delta^{13}C$ of all of the carbon depleted overnight and the $\delta^{13}C$ of the carbon fixed in the organic acid fraction is expressed as carbon isotope Δ. Exposed and shaded leaves were sampled on February 4, 1992 (wet season), and on March 16, 1992 (dry season).

	Wet Season		Dry Season	
	Exposed	Shaded	Exposed	Shaded
CO_2 fixed phase IV (1400–1800)	−28.3	−29.2	−24.6	−23.9
Carbon gained organic acids	−20.6		−16.1	−21.8
Carbon lost soluble sugars	−15.7 (67%)	−28.5	−14.0 (60%)	−17.7 (49%)
Carbon lost glucans	−21.0 (16%)	−28.1	−17.6 (14%)	−19.2 (20%)
Carbon lost starch	−22.8 (17%)	−25.0	−22.2 (14%)	−24.9 (20%)
External CO_2			−11.2 (11%)	−11.2 (11%)
Δ	2.8‰		0.9‰	3.1‰

which was citrate) were enriched in ^{13}C by 1.7‰ overnight. This supports the route of citrate synthesis shown in Figure 4a (Gout et al., 1993; Olivares et al., 1993). Thus, malate originally enriched in ^{13}C at the C-4 position via PEPC and malate dehydrogenase enters the mitochondrion, where fumarase randomizes ^{13}C between C-1 and C-4 of malate (Osmond et al., 1988). Malate is subsequently metabolized to oxaloacetate and condensed with acetyl-CoA to produce citrate with C-1 and C-6 enriched in ^{13}C (Gout et al., 1993). It is still unclear whether acetyl-CoA is produced from pyruvate formed from the glycolytic pathway or from the mitochondrial NAD^+-linked malic enzyme (Gout et al., 1993).

As CAM activity increased in the dry season and malic acid became the preferred organic acid accumulated at night, ^{13}C enrichment of this fraction increased. Moreover, estimates of Δ during the night, calculated from the difference between $\delta^{13}C$ of carbon exchanged between mobile pools of carbohydrate and organic acids (Table V), showed that in exposed plants Δ decreased from wet to dry season (i.e. 2.8–0.9‰) as CAM activity and malic acid accumulation increased. Shaded leaves with substantial CAM activity during the dry season had the highest malate:citrate ratio and the highest value of Δ (i.e. 3.1‰). However, these estimates of discrimination are somewhat lower than those measured directly by on-line fractionation during gas exchange, either for this population of *C. minor* (Borland et al., 1993) or for an epiphytic bromeliad (Griffiths et al., 1990). In plants in which respiratory recycling accounts for a significant proportion of the nighttime carbon budget (i.e. exposed leaves in wet and shaded leaves in the dry seasons), CO_2 uptake could be diffusion limited, which would lead to an increase in Δ (Borland et al., 1993). Alternatively, respiratory CO_2, having already been subjected to fractionation processes, would increase apparent discrimination when refixed at night (Griffiths et al., 1990; Borland et al., 1993).

Carbohydrate Partitioning during the Day-Night Cycle

Although it seems unlikely that different carbohydrate fractions form discrete pools of PEP destined either for respiration or organic acid synthesis at night in *C. minor*, there appears to be compartmentation between the products of C_3 and C_4 carboxylation during the light period. This was suggested by observations that carbon lost from soluble sugars during phase I of CAM was enriched in ^{13}C by 7 to 12‰ compared to CO_2 fixed during phase IV (Table V). These results could be explained by the existence of two soluble sugar pools, a vacuolar pool enriched in ^{13}C from the decarboxylation of organic acids and a rapidly turned over cytosolic pool of transport sugars, synthesized from CO_2 fixed during phase IV. Soluble sugars extracted in this study almost certainly were made up mainly of vacuolar sugars. It has been suggested that photosynthates produced during phase IV are used for the growth of CAM plants (Winter, 1985). Such compartmentation provides a means by which the metabolic pathways that constitute CAM and those that fuel growth can be regulated independently of one another.

In contrast to soluble sugars and glucans, carbon lost from the starch pool at night carried a more C_3-like isotopic signature. However, a flux of decarboxylation products into the starch pool cannot be excluded given that starch grains are synthesized by the successive deposition of layers. Thus, the carbon released at night from the degradation of the outermost layers of starch would be expected to carry a similar isotopic signature to the carbon fixed at the end of the preceding day, assuming no isotopic discrimination during starch synthesis.

From the results obtained here, and given that decarboxylation of organic acids during phase III is thought to be mediated via PEP carboxykinase in *Clusia* (Black, 1994), a scheme outlining possible carbon fluxes during phases III and IV in *C. minor* is proposed (Fig. 4b). It has been assumed that citrate is decarboxylated to the level of malate in the mitochondrion releasing 2 CO_2. Complete metabolism of citrate in the mitochondrion to release 6 CO_2 has been suggested as a possibility that would reduce photorespiration and may avoid photoinhibition (Haag-Kerwer et al., 1992). The scheme in Figure 4b illustrates that both glucans and starch and soluble sugars (shown as Suc) may be recovered during deacidification in *C. minor*. Coordination of chloroplastic and cytosolic metabolism may be mediated by high concentrations of the regulator molecule Fru-2,6-P_2 during phase III as shown for the PEP carboxykinase CAM plant *Ananas comosus* (Fahrendorf et al., 1987), in which soluble sugars also serve as the major source of PEP (Black et al., 1982). High concentrations of Fru-2,6-P_2 would be expected to inhibit Suc synthesis via Fru-1,6-bisphosphatase and promote starch/glucan synthesis in the chloroplast during phase III. At the same time, the flux of PEP toward Suc during decarboxylation could be mediated via PPI:Fru-6-P 1-phosphotransferase, which has a high activity in *A. comosus*

Table VI. *Energetics of malic and citric acid accumulation at night*

Two possible routes of glycolytic processing were considered for exposed and shaded leaves of C. *minor* sampled during the wet and dry seasons in Trinidad: (a) hexose units derived from starch were degraded via phosphofructokinase in the chloroplast and hexose units from soluble sugars were degraded via PPi:Fru-6-P 1-phosphotransferase (PPi-PFP) in the cytoplasm or (b) all hexoses were degraded by chloroplastic and cytoplasmic isozymes of phosphofructokinase. The ATP potentially available from dark respiration was calculated assuming a respiratory quotient of 1 and P:O ratio of 3. ATP potentially generated from citric acid accumulation was calculated assuming that half of the pool was derived from soluble sugars.

	(a) PFP (PPi)		(b) Phosphofructokinase (ATP)		Respiration
	Malate	Citrate	Malate	Citrate	
			mol ATP m^{-2}		
Wet season					
Exposed	−3.1	+344.5	−4.6	+306.8	+75.6
Shaded					+36.0
Dry season					
Exposed	−31.0	+225.6	−62.0	+208.0	+79.2
Shaded	−25.3	+67.6	−38.0	+62.4	+126.0

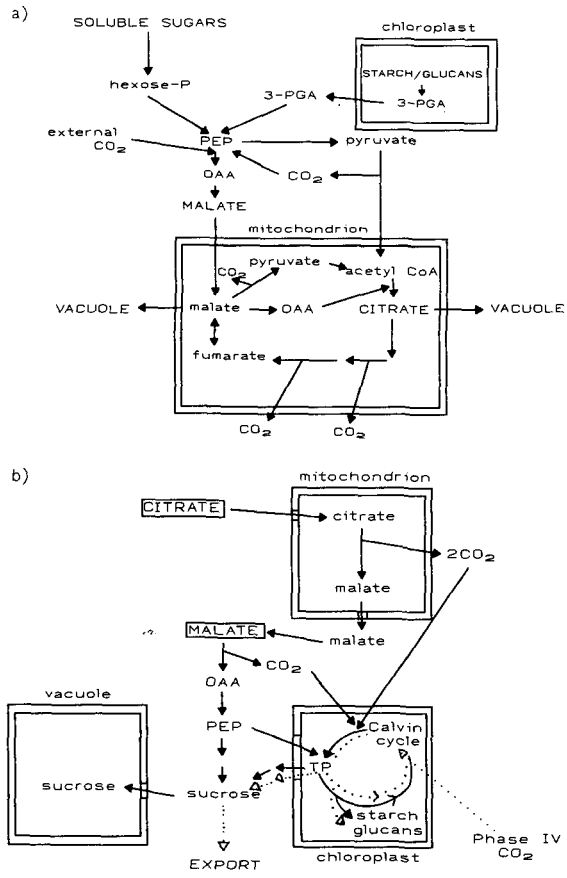

Figure 4. Carbon fluxes proposed for dark reactions of CAM (a) and decarboxylation (b) and phase IV carboxylation (. . . .) in C. *minor*. OAA, Oxaloacetate; 3-PGA, 3-phosphoglycerate.

(Carnal and Black, 1989) and which is promoted by Fru-2,6-P_2.

Seasonal Carbon Allocation to Growth and Regulation of CAM Induction

Up to 20% of daily net CO_2 uptake occurs during phase IV of CAM in C. *minor* (Borland et al., 1993) when CO_2 fixed directly via Rubisco will be used for synthesis of starch/glucans and Suc destined predominantly for export. Thus, exported Suc, which is subsequently used for growth, will carry a predominantly C_3 isotopic signature, regardless of the degree of CAM (Fig. 4b). This was reflected by the $\delta^{13}C$ of structural material, which was similar in leaves sampled during the wet and dry seasons.

The central role of carbohydrates in controlling the C_3-CAM switch is demonstrated by recent studies of *Mesembryanthemum crystallinum* in which the switch to CAM is accompanied by rapid 3- to 4-fold increases in starch turnover and in the activities of starch degradative enzymes prior to the transcription of the new PEPC isoform (Paul et al., 1993). For C. *minor*, factors that trigger the switch from C_3 to CAM result in the retention of more carbohydrates in leaves at the end of the day, possibly by reducing the flux of photosynthetically fixed carbon to transport Suc. However, the increase in storage carbohydrates could also occur at the expense of citrate, which declined significantly at the onset of the dry season. Such findings suggest a possible mechanism underlying suggestions that regulation of leaf-sap acidity via changes in citrate concentration (A.M. Borland, unpublished data) control the fast up and down regulation of CAM activity, which is characteristic of many *Clusia* species (Zotz and Winter, 1993).

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