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C_4 Pathway Photosynthesis at Low Temperature in Cold-tolerant *Atriplex* Species[1]

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ABSTRACT

Two species of *Atriplex* were grown under low temperature (8 C day/6 C night) and high temperature (28 C day/20 C night) regimes. The photosynthetic capacity of these plants was studied as a function of temperature in a leaf gas exchange cuvette. Both species showed substantial photosynthetic capacity between 4 and 10 C and this was not enhanced by growth at low temperatures but rather, was somewhat greater in plants grown at higher temperature. Photosynthetic capacity of low temperature-grown plants at high temperature was greater in *Atriplex confertifolia* (Torr. and Frem.) S. Watts., a native of cool deserts, than in *Atriplex vesicaria* (Hew. ex. Benth.) from warmer desert areas. Leaves of both species were also subjected to $^{14}CO_2$ pulse-chase and steady-state feeding experiments under controlled temperature conditions. These experiments revealed that the kinetics of carbon assimilation through the intermediates of the C_4 pathway is not substantially disrupted at low temperature in either species. There was, however, a substantial interchange of label between aspartate and malate at low temperature which was not evident at high temperature. There was also an increase in the pool sizes of the C_4 acids involved in photosynthesis of *A. confertifolia*. Speculation as to the explanation of these changes and their possible significance in promoting low temperature C_4 photosynthesis in these plants is presented.

The C_4 pathway of photosynthesis has been most commonly associated with plants of tropical or warm arid environments, a notion supported by recent statistical evaluation of the percentage of C_4 species in regional grass and dicotyledon floras in North America in relation to climatic factors (27, 31). These studies indicate that the occurrence of C_4 grasses is best correlated with night temperature during the growing season and that the occurrence of C_4 dicotyledons is best correlated with summer pan evaporation. It would be difficult to assess the performance, as opposed to the occurrence, in such a comprehensive way, but the few published studies of C_4 photosynthesis as a function of temperature further indicate that most of the grasses examined are low temperature-limited. Thus, several C_4 tropical grasses show low temperature compensation points between 6.5 and 10 C (18) and many are chilling-sensitive, showing impaired photosynthesis and Chl breakdown after relatively brief exposure to temperatures to 10 C (20, 24, 30). A C_4 dicotyledon, *Tidestromia oblongifolia*, which grows only under high temperature conditions, also shows impaired photosynthesis below 20 C (2). On the other hand, C_4 dicotyledons from cool, oceanic environments and from cool desert areas show substantial net CO_2 fixation at temperatures below 10 C, although the temperature optimum for photosynthesis may be 25 to 30 C. *Atriplex sabulosa* from northern Europe (2) and *Atriplex confertifolia* from the North American Great Basin (4, 22, 33) share these properties, as does the C_4 grass, *Spartina townsendii*, from cool, coastal environments (17). Indeed, there is no fundamental reason why C_4 pathway photosynthesis should exclude a species from cool regions (2).

The chilling-sensitive, low temperature-limited C_4 grasses and dicots investigated to date all appear to be of the "NADP malic enzyme" type C_4 plants (8, 9, 11). When treated at low temperature, such plants show substantial changes in the kinetics of $^{14}CO_2$ assimilation via the C_4 pathway, and marked changes in chloroplast ultrastructure and properties (4, 25, 29). The low temperature-tolerant C_4 plants, such as *Atriplex* species, appear to be mostly of the "NAD malic enzyme" type (8, 11). We have compared photosynthesis and $^{14}CO_2$ radiotracer kinetics of photosynthesis at low and high temperature in leaves of *A. confertifolia* and *A. vesicaria*, from plants grown at low and high temperatures. We present evidence that low temperatures modify the kinetics of C_4 pathway carbon assimilation, even in these low temperature-tolerant C_4 plants, but do not appear to disrupt the metabolic sequence in the manner observed in chilling-sensitive C_4 plants.

MATERIALS AND METHODS

Plant Material. *A. vesicaria* Hew. ex. Benth. was cultivated from seed collected near Deniliquin, N.S.W., Australia (35°35'S, 145°2'E, 95 m elev.). *A. confertifolia* (Torr. and Frem.) S. Wats. was cultivated either from seed or cuttings from shrubs in Curlew Valley of northern Utah (41°52'N, 113°5'W, 1350 m elev.). Germination and seedling establishment took place in a controlled temperature greenhouse in the CSIRO Phytotron in Canberra at 27/22 C day/night temperatures under natural solar illumination. After establishment in the greenhouse for a period of 3 to 4 weeks, the plants were moved to controlled environment chambers in the Phytotron with illumination by fluorescent and incandescent lights at the highest level available in these cabinets (800-900 μeinsteins$\cdot m^{-2}\cdot sec^{-1}$ 400-700 nm). The plants to be grown at high temperature were moved directly into a cabinet at 28/20 C while the plants to be acclimated at low temperature were moved into cabinets in which the temperature was gradually lowered (5 C each 10 to 14 days) until the low temperature regime of 8/6 C was reached. Plants were maintained at the desired growth temperatures for a minimum of 4 weeks before being used in the experiments. Both the state of development and morphology of the plants varied considerably

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with growth temperature so that it was neither convenient nor meaningful to use leaves of the same chronological age in the photosynthetic assays or $^{14}CO_2$-labeling experiments. In any case, all measurements were conducted on young plants which were still producing new leaf material and were not in the reproductive stage. All the plants were grown in a perlite-vermiculite mixture and irrigated with aerated Hoagland solution once a day and tap water once a day. In the controlled environment cabinets, the thermoperiod and fluorescent lamps were set for a 12-hr day and the incandescent lamps were illuminated for 16 hr.

Plant Gas Exchange Measurements. Net photosynthesis and transpiration were simultaneously measured in a gas exchange cuvette which was described earlier (16, 26). Several leaves of these microphyllous plants were used rather than a single leaf. Irradiation was provided by a metal halide lamp at 1000 ± 50 μeinsteins$\cdot m^{-2}\cdot sec^{-1}$ (400–700 nm). Leaf temperatures were measured with fine wire thermocouples appressed to the underside of the leaves. Gas exchange rates were normally determined at ambient CO_2 concentrations of about 525 ng$\cdot cm^{-3}$ but in several experiments a range of ambient CO_2 concentrations from 0 to 500 ng$\cdot cm^{-3}$ was used.

The equation for CO_2 flux into the leaf intercellular spaces is

$$P = \frac{C_a - C_i}{r_s' + r_a'} \quad (1)$$

where C_a and C_i are CO_2 concentrations in the air surrounding the leaf and in the leaf intercellular spaces, respectively; r_s' and r_a' are stomatal and boundary layer resistances for CO_2; and P is net photosynthetic rate. Leaf resistances for H_2O vapor were determined from leaf temperatures, transpiration rates, and absolute humidity of the air and converted to CO_2 diffusion resistance (19).

In order to compare photosynthetic capacity of leaves in which stomatal diffusion resistance differed, the measured photosynthetic rates were adjusted to account for differences in C_i. Rather than assume a linear response of P to C_i between the CO_2 compensation point and concentrations approaching ambient levels (6, 7), photosynthetic rates as a function of C_a were determined in the cuvette system and this was then related to P as a function of C_i, where C_i was determined by rearrangement of equation 1. The photosynthetic response curve for *A. vesicaria* at 23 C as a function of C_i is shown in Figure 1 and is typical of the curves found for other C_4 plants.

The adjustment of photosynthetic rate to account for differences in stomatal diffusion resistance was made as follows. The adjusted photosynthetic capacity, P' is

$$P' = P + \Delta P \quad (2)$$

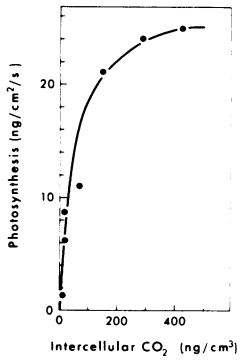

FIG. 1. Net photosynthesis of an *A. vesicaria* plant as a function of calculated intercellular CO_2 concentration. This individual was grown at 28/20 C day/night temperature regime and assayed for photosynthesis at 23 C.

where the change in photosynthetic rate, ΔP, is a function of change of intercellular CO_2 concentration, ΔC_i, following correction for leaf diffusive resistances and the slope, f, of the curve P *versus* C_i (Fig. 1) over the relevant range of C_i, *i.e.* $\Delta P = f(\Delta C)$. Equations 1 and 2 were combined to solve for P' at the adjusted intercellular CO_2 concentration C_i' at the chosen leaf resistances ($r_s'' + r_a''$), according to

$$P' = P\frac{(1 + f[r_s' + r_a'])}{(1 + f[r_s'' + r_a''])} \quad (3)$$

In this study, calculated values of P' usually involved small values of ΔP (equation 2) since the dependency of P on C_i was not great in the range of C_i affected by adjustment of leaf diffusion resistance.

Determination of Short Term Photosynthetic Products. Continuous feeding and pulse-chase experiments with $^{14}CO_2$ were conducted using a 3-liter, temperature-controlled chamber, fitted with small fans and a release mechanism to flush rapidly the air surrounding the leaves with $^{12}CO_2$ during pulse-chase. Leaf temperatures, measured with fine wire thermocouples, showed only about 1 C transient change during the pulse-chase manipulations. The system could accommodate six small branches each containing up to 10 small leaves. The cuvette was illuminated with a xenon arc lamp which irradiated the shoots at 900 μeinsteins$\cdot m^{-2}\cdot sec^{-1}$ (400–700 nm). Before exposure to $^{14}CO_2$, the plants were pre-conditioned for 30 min using an air stream of 373 μl/l CO_2 in air. The same air stream was used during the chase period. For the labeling, approximately 100 to 200 μCi of $^{14}CO_2$ (6 μCi/μmol) were used (28). During the course of the continuous feeding or pulse-chase experiments, 1-ml samples of the air in the labeling cuvette were taken and the $^{14}CO_2$ adsorbed in 20% (v/v) ethanolamine in methanol for determination of specific radioactivity. At pre-set times during the feeding or chase periods, individual shoots were removed and dropped immediately into boiling 80% (v/v) ethanol.

Extraction in boiling ethanol continued until pigments had been removed. Leaves were then excised, stems removed, and the leaves were ground in a glass homogenizer. The homogenate was further extracted in boiling water, cooled, and removed by centrifugation. The residual pellet was washed by resuspension in water and again centrifuged. The combined supernatant extracts were made to volume. An aliquot of the soluble extract was counted in scintillation fluid and a further aliquot extracted with chloroform then into acetone for Chl determination by the pheophytin method (32). The insoluble residue was again suspended in water and aliquots of the suspension counted in scintillation fluid.

The bulk of the soluble extract was separated into an amino acid fraction by passage through Dowex AG-50, W-X8, 100 to 200 mesh, and another fraction containing carboxylic acids, phosphorylated compounds, and neutral compounds. The amino acids were recovered by elution with 10% (v/v) NH_4OH and aliquots of each fraction were counted in scintillation fluid. Recovery of ^{14}C was better than 95%. These fractions were dried under vacuum and aliquots of the amino acid fraction subjected to paper electrophoresis in acetic acid-formic acid buffer (15:5 v/v) at 2,500 v. The other fraction was subjected to two-dimensional paper chromatography on sheets (20 × 20 cm), using ethanol-ammonia-water (4:1:1, v/v) in the first, and diethyl ether-formic acid (98%)-water (7:2:1, v/v) in the second dimension (21). Labeled compounds were located by autoradiography, cut from the chromatograms, and counted in scintillation fluid. The total label on the chromatogram was assessed and that in each compound expressed either as a percentage of the total fixed or as μmol$\cdot mg^{-1}$ Chl, on the basis of the specific radioactivity of the $^{14}CO_2$ supplied and the Chl content of the extract.

Larger scale paper electrophoresis or single dimension paper

chromatography in ethanol-ammonia-water was used to purify the labeled malic and aspartic acid from the fractionated extracts. The labeled compounds were located by autoradiography, eluted from the paper in 0.2 to 0.5 ml water, and stored frozen. The concentration of malate in these preparations was measured (14) and label in the C-4 carboxyl carbon of malic acid was estimated after decarboxylation with partly purified maize leaf NADP malic enzyme as described earlier (2) except that the reaction was stopped with 0.5% (v/v) hydroxylamine hydrochloride in 0.5 N HCl and residual $^{14}CO_2$ was removed by purging with CO_2 gas. Authentic uniformly labeled malate released 21 to 25% $^{14}CO_2$ and enzymically prepared [4-^{14}C]malate (10) released 98% $^{14}CO_2$ in controls run with each experiment. The C-4 carboxyl carbon of [^{14}C]aspartate was released as $^{14}CO_2$ after conversion to [^{14}C]oxaloacetate with aspartate aminotransferase in the presence of Mn^{2+} (23). The aspartate degradations were run at 37 C for 6 hr and then allowed to stand overnight at 20 C before acidification with acid hydroxylamine hydrochloride. [4-^{14}C]Aspartate, prepared enzymically, regularly yielded 93% $^{14}CO_2$ and [U-^{14}C]aspartate yielded 24 to 33% $^{14}CO_2$ in controls run with each experiment. No corrections for the recovery of label in the C-4 carboxyl of standards have been applied to the experimental data.

RESULTS

TEMPERATURE EFFECTS ON LEAF PROPERTIES

Growth of both *Atriplex* species was rather slower at 8/6 C than at 23/20 C and low temperature-grown plants had a more upright habit and larger leaves than those grown at high temperature. Leaves of *A. vesicaria* were much thicker when plants were grown at low temperature, but there was no pronounced difference in leaf thickness of low temperature and high temperature-grown *A. confertifolia*. The differences in leaf thickness in *A. vesicaria* are shown in the transverse section light micrographs of Figure 2 which indicate that mature leaves of the low temperature-grown plants were about 1.7 times thicker than those of high temperature-grown plants. This difference is consistent with the 1.6 times greater ratio of dry weight to leaf area for these leaves (Table I). Growth temperature had little effect on this ratio in *A. confertifolia*.

Even at the low magnification of Figure 2, the swollen, starch-containing chloroplasts of both mesophyll and bundle sheath cell chloroplasts in leaves of low temperature-grown plants are evident, and this observation is confirmed in the electron micrograph shown in Figure 3. Starch grains were not observed in mesophyll cell chloroplasts in leaves from high temperature-grown *A. vesicaria*. This suggests that cold-tolerant C_4 plants may not differ from chilling-sensitive C_4 species in respect to starch accumulation at low temperatures (13). Chl content of leaves, expressed on a dry weight or leaf area basis, was not much affected by growth temperature in either *A. vesicaria* or *A. confertifolia* (Table I).

PHOTOSYNTHETIC CO_2 ASSIMILATION CAPACITY

Despite the different thermal environments from which these species were derived, the photosynthetic capacities of the *Atriplex* species were remarkably similar (Fig. 4). *Atriplex confertifolia* from the cool desert environment did not exhibit higher photosynthesis at low temperatures. Both species were successfully grown at the low temperature regime, but growing these plants at low temperature did not enhance photosynthetic capacity at low temperatures. Only at the lowest temperature attainable in this cuvette system (3–5 C) were the photosynthetic capacities of the plants grown at low temperature equal to those of plants grown at high temperatures (Fig. 4). Even then, when photosynthetic capacities of the two species were adjusted to a

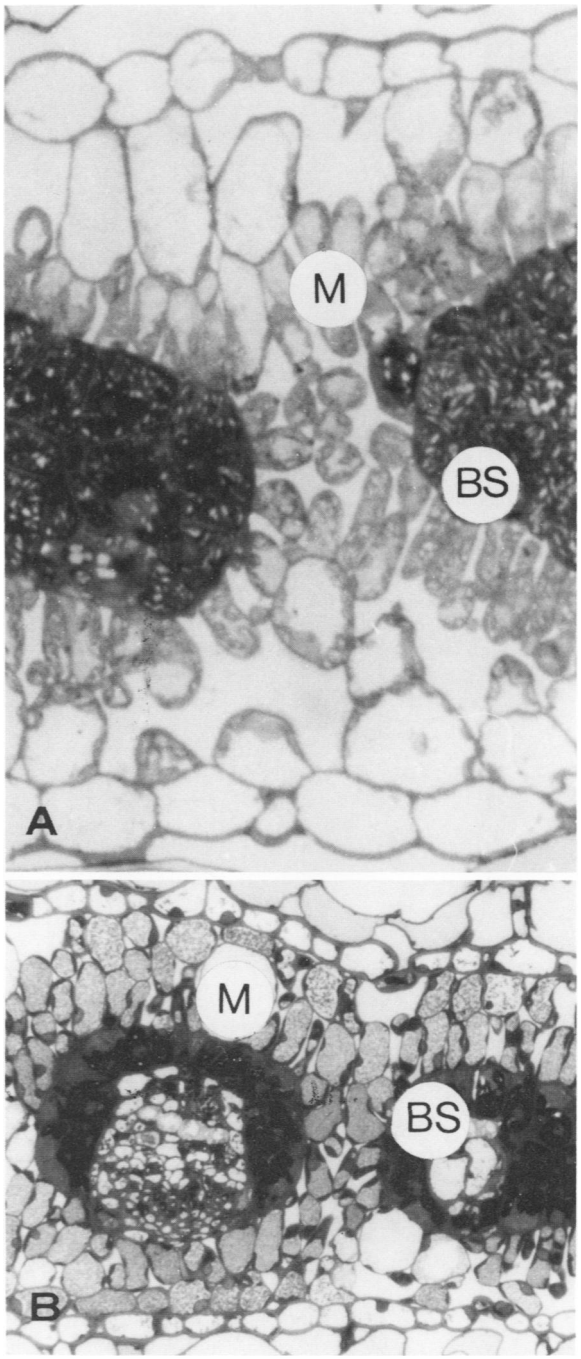

FIG. 2. Thin section of leaves from *A. vesicaria* grown at 8/6 C (A) and at 28/20 C (B). Mesophyll cells (M) and bundle sheath cells (BS); light micrographs × 100.

Table I. Effect of growth under different temperature regimes on properties of Atriplex leaves used in $^{14}CO_2$ and gas exchange experiments

Species and Growth Temperature		Fresh wt. / Dry wt.	Dry wt. / Area	Chlorophyll Content: Weight	Chlorophyll Content: Area
			mg/cm^2	mg/g dry wt	mg/cm^2
A. vesicaria	8/6 C	4.9 (3)[1]	13.2 (3)	5.85 (2)	0.076
	28/20 C	6.0 (2)	8.5 (4)	8.10 (3)	0.069
A. confertifolia	8/6 C	6.9 (2)	8.8 (3)	4.54 (2)	0.040
	28/20 C	6.1 (1)	8.7 (2)	5.72 (2)	0.050

[1] Number of measurements shown in parentheses.

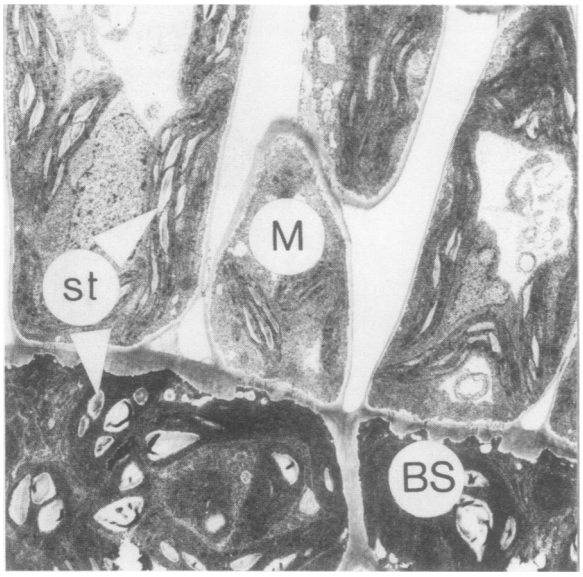

FIG. 3. Thin section of leaf from *A. vesicaria* grown at 8/6 C showing mesophyll cells (M) and bundle sheath cells (BS), both of which contain chloroplasts with large starch grains (st). Electron micrograph × 1,600.

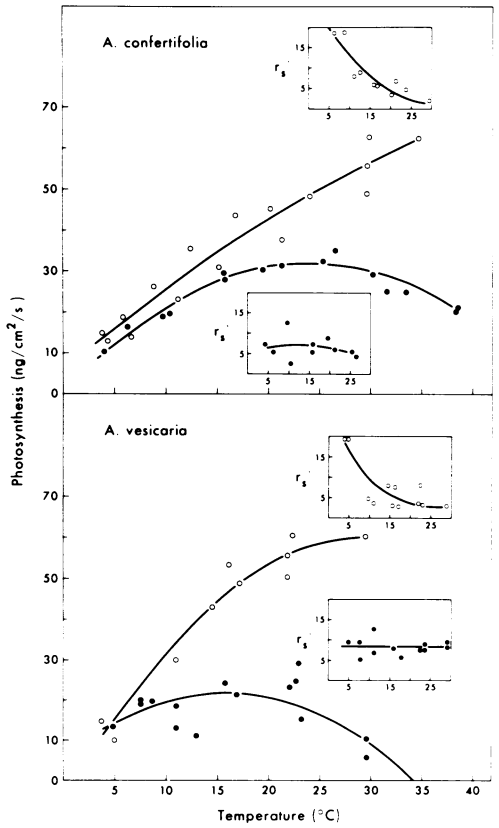

FIG. 4. Net photosynthesis as a function of assay temperature for *A. vesicaria* and *A. confertifolia* grown at day/night temperature regimes of 28/20 C (○) and at 8/6 C (●). Corresponding leaf diffusion resistances for CO_2, r_s', are shown as a function of assay temperature. This resistance includes a small boundary layer component. Individual measurement values for two to five individual plants are shown for each condition. The curves are derived from least squares fits to the data.

common leaf resistance of 8 sec·cm^{-1} (a median value for these species at lower temperatures, Fig. 4), the photosynthetic capacities of high temperature-grown plants exceeded those of low temperature-grown plants when assayed at 4 and 10 C (Table II).

The only readily apparent difference in photosynthetic capacity between *A. vesicaria* and *A. confertifolia* was the greater photosynthetic rate of *A. confertifolia* at high temperatures when these species were grown at low temperature. This trend is evident in Figure 4 but is better illustrated by the adjusted photosynthetic capacities. When adjusted for a common leaf diffusion resistance of 5 sec·cm^{-1} (a median value for these species at high temperatures, Fig. 4), the low temperature-grown *A. confertifolia* exhibited three times the photosynthetic capacity at 30 C when compared to *A. vesicaria* grown at low temperature (30 and 10 ng·cm^{-2}·sec^{-1}, respectively). The adjusted rates at 30 C were not, however, substantially different when the plants were grown at high temperatures, *i.e.*, 55 and 59 ng·cm^{-2}·sec^{-1} for *A. confertifolia* and *A. vesicaria*, respectively.

RADIOTRACER KINETIC EXPERIMENTS

Time Course of $^{14}CO_2$ Fixation. The volume of the $^{14}CO_2$ exposure chamber used in these experiments was sufficient to ensure that the rate of $^{14}CO_2$ fixation remained linear throughout the experiment. The addition of $^{14}CO_2$ to the chamber increased the total CO_2 concentration by less than 5% and the depletion of $^{14}CO_2$ during the longest experiments did not exceed 40%. As shown in Figure 1, CO_2 fixation by these *Atriplex* species is saturated in the vicinity of 300 ng·cm^{-3} (152 μl·l^{-1}) intercellular CO_2 concentration. The ambient CO_2 concentration of the chamber did not decline below approximately 240 μl·l^{-1} and the time course (Fig. 5) showed that $^{14}CO_2$ fixation was linear with time. The scatter of points is largely due to the indirect method of Chl estimation used, and the variable proportion of leaves of differing age and Chl content on each shoot. In the pulse-chase experiments, the $^{12}CO_2$-flushing technique used removed all of the $^{14}CO_2$ in less than 5 sec so that, on a Chl basis, the radioactivity fixed remained constant throughout the chase period (Fig. 5). The rates of $^{14}CO_2$ fixation, calculated on the

Table II. Low temperature photosynthetic capacity of Atriplex species grown at two temperature regimes, adjusted to a common leaf diffusion resistance of 8 sec·cm^{-1}

Species and Growth Temperature		Leaf Temperature During Assay	Adjusted Photosynthesis (P')
		° C	ng·cm^{-2}·sec^{-1}
A. vesicaria	28/20 C	4	13.1
		10	31.9
	8/6 C	4	12.6
		10	19.6
A. confertifolia	28/20 C	4	15.5
		10	26.7
	8/6 C	4	10.4
		10	21.1

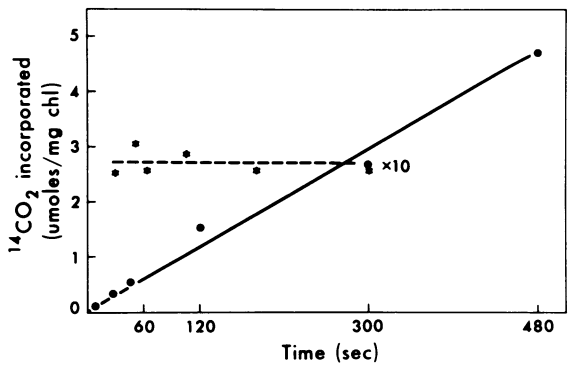

FIG. 5. Time course of $^{14}CO_2$ incorporation in shoots of *A. vesicaria* at 9.4 C. Steady-state experiment (●) and pulse-chase experiment after 30-sec exposure to $^{14}CO_2$ (*).

Table III. Effect of growth temperature and temperature during exposure to $^{14}CO_2$ on the labeling of C_4 dicarboxylic acids in *Atriplex* species

Exposure Time, Species and Growth Temperatures		Distribution of ^{14}C at 7.5 C and 27.5 C			
		Malate	Aspartate	Malate	Aspartate
10 sec $^{14}CO_2$ exposure			%		
A. vesicaria	8/6 C	19	51	36	43
	28/20 C	39	46	41	37
A. confertifolia	8/6 C	22	41	-	-
	28/20 C	-	-	49	32
30 sec $^{14}CO_2$ exposure					
A. vesicaria	8/6 C	28	41	-	-
	28/20 C	-	-	34	27
A. confertifolia	8/6 C	24	49	-	-
	28/20 C	-	-	37	29

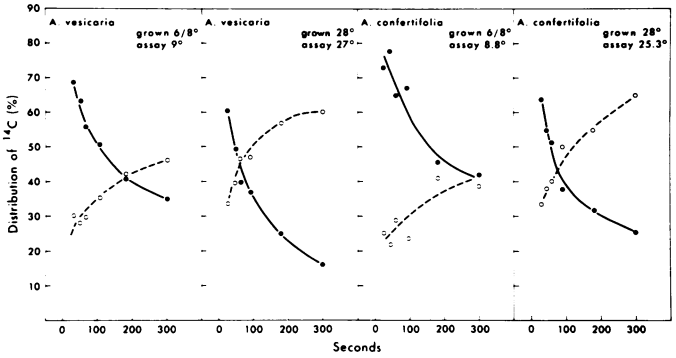

FIG. 6. Relationship between label in C_4 dicarboxylic acids (●) and 3-PGA and its products (○) during pulse-chase experiments after 30-sec exposure to $^{14}CO_2$ in *Atriplex* species at different temperatures.

basis of the initial sample in pulse-chase experiments, always agreed closely with those calculated from the slope of the time course curve for the same species under the same conditions. The rates of $^{14}CO_2$ fixation in different radiotracer experiments were similar to those estimated from the gas exchange experiments.

Short Term Labeling of C_4 Dicarboxylic Acids. In all experiments with both species grown under both temperature regimes, the C_4 acids malate and aspartate comprised the principal initial products of $^{14}CO_2$ fixation. Table III shows the percentage of ^{14}C found in these two compounds after 10 and 30 sec $^{14}CO_2$ fixation by both species under different conditions. In all cases, plants grown at low temperature and exposed to $^{14}CO_2$ at low temperature showed substantially more label in aspartate than malate when compared with plants grown at high temperature and exposed to $^{14}CO_2$ at high temperature. When low temperature grown *A. vesicaria* was assayed at high temperature, the percentage label in malate was higher and that in aspartate was lower after 10 sec exposure to $^{14}CO_2$ than in the same plants assayed at low temperature. A similar but less pronounced change in the distribution of C_4 acid label was observed when high temperature-grown *A. vesicaria* plants were assayed at low temperature (Table III). These data suggest that assay temperature can modify the patterns of C_4 acid distribution of *A. vesicaria* from different growth temperature regimes.

Pulse-Chase Labeling Experiments. Figure 6 shows the relationship between the label in C_4 dicarboxylic acids (malate and aspartate) and the label in 3-PGA[3] and its products (3-PGA, phosphorylated compounds, sugars, and insoluble compounds) during 30-sec $^{14}CO_2$ pulse, 270-sec $^{12}CO_2$ chase experiments for both *Atriplex* species at both temperatures. In each case there was a rapid initial loss of label from the C_4 dicarboxylic acids and an increase in label in 3-PGA and its products.

Figure 6 suggests that the rate of transfer of label from C_4 acids to 3-PGA and its products is slower at low temperatures than at high temperatures. This is confirmed in the more detailed pulse-chase data shown in Figure 7. These data show that the slower rate of ^{14}C transfer from C_4 acids at low temperature is associated with an early increase of ^{14}C in malate at the same time as a loss in label from aspartate. This trend was observed in both species but is more pronounced in *A. confertifolia*. At the same time, the slower transfer of label into 3-PGA and its products at low temperature, although not evident in the 3-PGA + glycerate component, is obvious in the slower movement of label into phosphorylated compounds, sugars, and insoluble

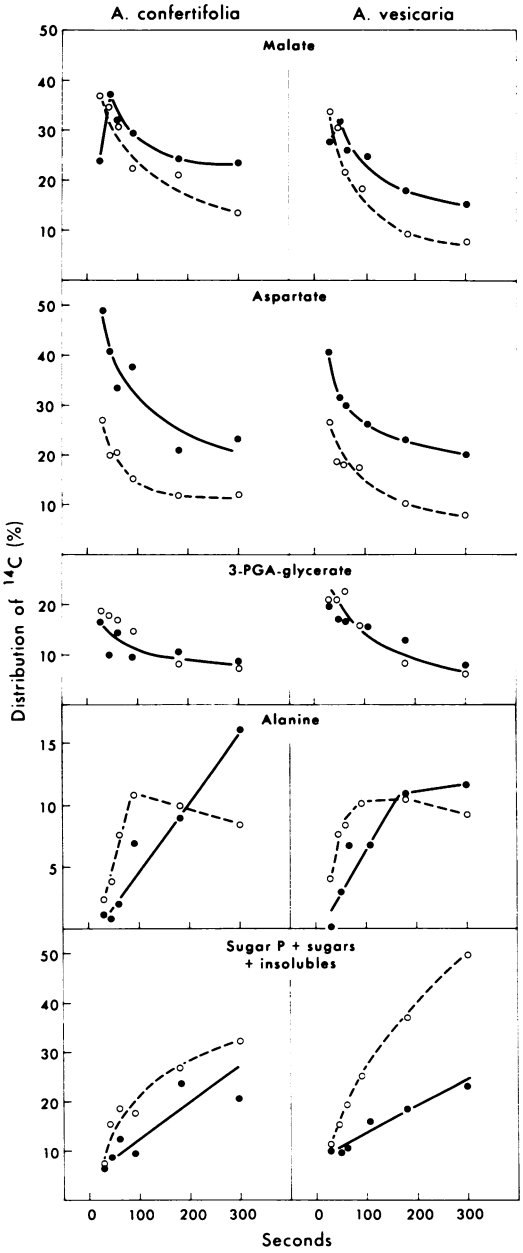

FIG. 7. Changes in the distribution of label among intermediates of C_4 photosynthesis in *Atriplex* species during pulse-chase experiments after exposure to $^{14}CO_2$ for 30 sec at about 27 C (○) and at about 9 C (●).

[3] Abbreviation: 3-PGA: 3- phosphoglycerate.

materials. Of particular interest is the slower labeling of alanine at low temperature in both species. Figure 7 shows that the transfer of label from aspartate and from 3-PGA + glycerate was not substantially influenced by differences in temperature.

Steady-state $^{14}CO_2$ Assimilation Experiments. Longer term, steady-state $^{14}CO_2$ assimilation experiments confirm the trends evident in the $^{14}CO_2$ pulse-chase experiments. In the early stages of carbon assimilation at low temperature in both species there was a substantial increase in the proportion of label in malate, at which time the proportion of label in aspartate was declining (Fig. 8). This trend, again somewhat more evident in *A. confertifolia* than in *A. vesicaria,* is presumably a manifestation of the transfer of label from aspartate to malate at low temperature observed in pulse-chase experiments (Fig. 7). Both experimental approaches give no indication of this process at high temperature. Figure 8 also shows that the proportion of label in sugars and insoluble compounds increased more rapidly at high temperature than at low temperature in both species. These changes are associated with a decline in the proportion of label in phosphorylated compounds, excluding 3-PGA. The proportion of label in 3-PGA + glycerate in both species increased throughout the low temperature experiments and for the greater part of the high temperature experiments. This was largely due to increased 3-PGA label which tended to parallel the label in alanine.

The principal objective of the steady-state $^{14}CO_2$ assimilation experiments was to measure the pool sizes of C_4 acids involved in photosynthetic metabolism under different temperature regimes. The pool sizes were estimated from the amount of radioactivity in the C-4 carboxyl carbon for the C_4 acids. It was assumed that this carbon would be the first to saturate with ^{14}C and the first to attain the same specific radioactivity as the $^{14}CO_2$ supplied (12). At saturation, the pool of malate or aspartate involved in photosynthetic turnover can be estimated by dividing the radioactivity in the C-4 carboxyl carbon (dpm/mg Chl) by the measured specific radioactivity of the $^{14}CO_2$ supplied (dpm/μg atom C = dpm/μmol CO_2). Operationally, the total radioactivity found in malate or aspartate at a given time was corrected by the percentage found to be associated with the C-4 carboxyl carbon following degradation and then divided by the average specific radioactivity measured during the first 10 to 15 sec of the experiment. Figure 9 shows the estimated percentage of ^{14}C associated with the C-4 carboxyl carbon in the course of one experiment. It is evident that even after 300 sec, more than 50% of the total ^{14}C in malate and aspartate is confined to the C-4 carboxyl carbon and that other carbon atoms of these acids are still far from total saturation with ^{14}C. However, the very similar behavior of the two acids in Figure 9 indicates that they come rapidly into equilibrium.

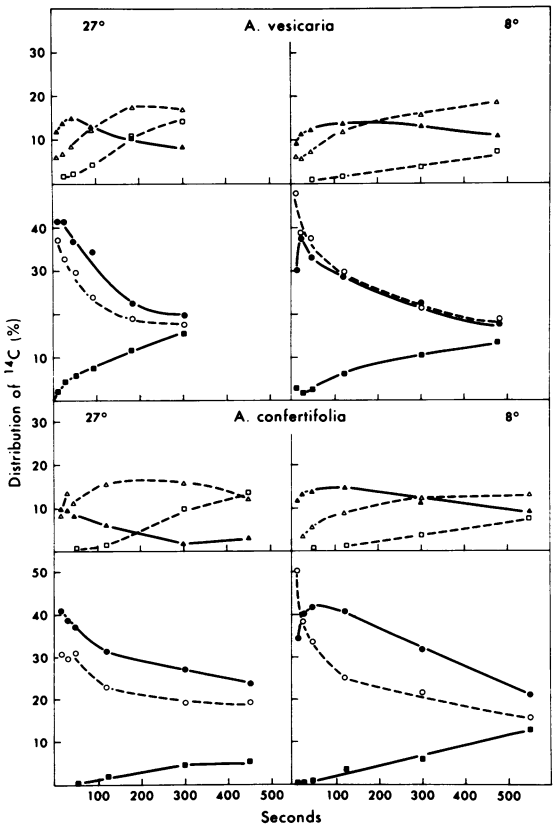

FIG. 8. Changes in the distribution of label among intermediates of C_4 photosynthesis in *Atriplex* species during steady-state exposure to $^{14}CO_2$ at 27 C and 8 C. Aspartate (O- - -O); malate (●——●); alanine (■——■); 3-PGA + glycerate (△- - -△); phosphorylated sugars (▲——▲) and sugars + insoluble compounds (□- - -□).

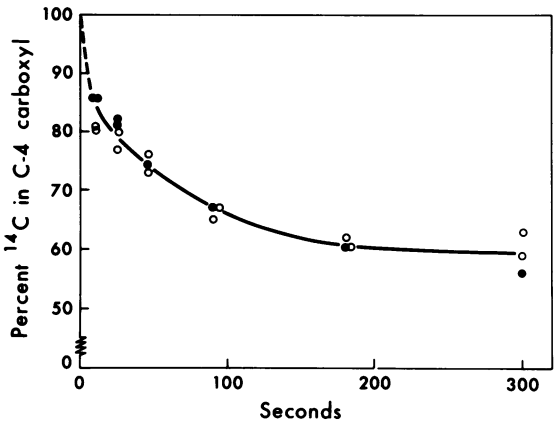

FIG. 9. Changes in the per cent of total ^{14}C in the C-4 carboxyl carbon of malate (●) and aspartate (O) isolated from leaves of *A. vesicaria* during steady-state $^{14}CO_2$ fixation at 27 C.

The estimated concentration of ^{14}C in the C-4 carboxyl carbon of malate and aspartate in all steady-state labeling experiments analyzed is shown in Figure 10. These data suggest that the carboxyl carbons of both acids approach saturation with $^{14}CO_2$. In all cases, the approach to saturation is more rapid at high temperature than at low temperature. These estimates of concentration of ^{14}C in the C-4 carboxyl carbon at saturation may be taken as equivalent to the size of C_4 acid pools involved in photosynthetic metabolism. Figure 10 thus suggests that in *A. vesicaria* there is little effect of low temperature on the size of the C_4 acid pools involved in photosynthesis and that the pools of malate and aspartate are similar in size (about 0.5 μmol/mg Chl). In *A. confertifolia,* however, malate pools are about twice the size of the aspartate pools and at low temperatures the pool of either C_4 acid is about twice as large as that at low temperature.

DISCUSSION

The above data show that these C_4 *Atriplex* species are capable of photosynthesis at low temperature and that the kinetics of carbon assimilation through the intermediates of the C_4 pathway are not substantially disrupted at low temperature. The temperature response curves for photosynthesis in these plants grown at low temperature are similar to those for C_3 and C_4 *Atriplex* species of cool coastal environments and unlike those for cold-sensitive C_4 plants (2, 30). *A. confertifolia,* native to cool deserts and grown here at low temperature, showed a greater photosynthetic capacity than *A. vesicaria* grown under the same conditions when both species were assayed at high temperatures. This observation suggests that C_4 photosynthesis in *A. confertifolia* is

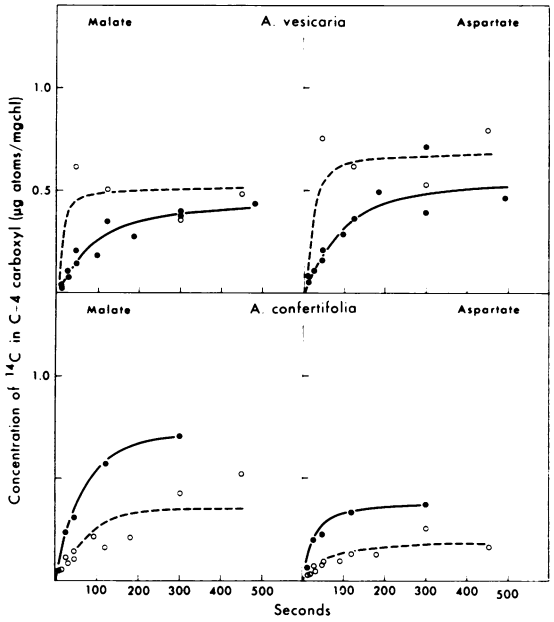

FIG. 10. Estimates of the concentration of labeled malate and aspartate involved in steady-state $^{14}CO_2$ assimilation in *Atriplex* species at about 27 C (●) and about 9 C (○). (Calculated from the ^{14}C content of the C-4 carboxyl carbon and specific radioactivity of $^{14}CO_2$ supplied.)

better suited to progression from low to high temperatures than is C_4 photosynthesis in *A. vesicaria*. Field observations show that *A. confertifolia* is most active in the spring (average April-May temperature, 9 C, Curlew Valley, Utah) but retains some photosynthetic capacity well into the hot summer months in its native habitat (5, 33). The performance of low temperature-grown *A. confertifolia* in the laboratory is consistent with these field observations. Contrary to our expectations, the photosynthetic capacity of *A. vesicaria,* native to warm deserts and grown here at high temperature, was not abruptly curtailed when assayed at low temperature. This species is certainly active following rain in summer months (average December-February temperature, 23 C, Deniliquin, New South Wales, Australia) but may also be active in cooler parts of the year.

Both *Atriplex* species showed evidence of some degree of photosynthetic acclimation to growth temperature. The temperature optimum for photosynthesis in ambient air is clearly lower in low temperature-grown plants than high temperature-grown plants (Fig. 4), but the absence of enhanced photosynthetic capacity at low temperatures is conspicuous (Fig. 4, Table II). The photosynthetic capacity at low temperatures in cold-tolerant C_3 and C_4 plants is usually enhanced by growth at lower temperatures (1, 2, 22). However, we have not found reports of C_4 species being cultured at such low temperatures. Growth of these plants at an intermediate temperature range (*e.g.* 10–15 C) might enhance photosynthetic capacity at temperatures below 10 C. It is apparent, however, that growth of these plants at the 28/20 C temperature regime did not result in a marked curtailment of photosynthetic capacity at lower temperatures.

The *in vivo* radiotracer kinetic experiments conducted at controlled temperatures indicated that the principal features of C_4 photosynthesis were retained in leaves of both *Atriplex* species when grown and assayed at low and high temperatures. For example, there is little indication that the precursor-product relationship between C_4 acids and 3-PGA and its products was influenced by growth or assay temperature (Fig. 6), or that the labeling of the C_4 acids involved anything more than the primary carboxylation of PEP (Fig. 9). The experiments do, however, show several related effects of low temperature on the radiotracer kinetics of key intermediates of the C_4 pathway. The data are consistent with the following progression of events.

At low temperature the transfer of label from C_4 acids to 3-PGA and its products is slower (Figs. 6–8). Correspondingly, the transfer of label to reduced products of the photosynthetic carbon reduction cycle is slower at low temperature and the labeling of alanine, which reflects the C_3 residue released during the transfer of label from C_4 acids to 3-PGA (12), is also slower. These related kinetic changes are consistent with current formulations of integrated C_4 pathway photosynthesis (3, 12) and are consistent with the reduced rates of CO_2 assimilation observed at low temperature (Fig. 4).

At low temperature the slower transfer of label from C_4 acids is associated with an interchange of labeled carbon between aspartate and malate. At low temperatures label appears to accumulate transiently in malate (Figs. 7 and 8). This "backing-up" of label in malate occurs before decarboxylation, at a time when both C_4 acids are predominantly labeled in the C-4 carboxyl carbon. It is evident in both species but most pronounced in *A. confertifolia*.

At low temperatures, the more pronounced backing-up of label in malate in *A. confertifolia* is consistent with the larger pool size of photosynthetic malate, relative to aspartate, in this species compared with *A. vesicaria*. Both C_4 acid pools increase in size in *A. confertifolia* grown at low temperatures (Fig. 10) whereas those in *A. vesicaria* were unchanged.

These phenomena appear to be internally consistent in different experiments and between species. The remainder of the discussion is addressed to the interpretation of these phenomena on the basis of our understanding of the NAD malic enzyme type C_4 pathway in these plants, how they differ from the low temperature radiotracer kinetics of chilling-sensitive NADP malic enzyme type C_4 plants, and whether they indicate processes which might account adequately for normal photosynthetic performance of these C_4 plants at low temperatures.

In *Atriplex* species and other NAD malic enzyme type C_4 plants, aspartate is the principal initial product of CO_2 fixation in mesophyll cells but this is converted to malate in bundle sheath cell mitochondria prior to decarboxylation (12). Whether the backing-up of labeled malate observed above takes place in mesophyll cells, bundle sheath cells, or specifically in bundle sheath mitochondria, or in all three sites, is not clear. Although the activity of NADP malate dehydrogenase in mesophyll cells of these plants is low (12), it is likely that the additional NADPH required for the net conversion of aspartate to malate would be readily available in these cells. The possibility of some direct labeling of malate in these cells should not be ruled out. It is difficult to imagine a source of NADH which would allow the backing-up of labeled malate to take place in bundle sheath cell mitochondria. The aspartate to malate conversion in these organelles is a tightly self-coupled process (12) and net synthesis of malate requires additional inputs of NADH.

The location of the increased pool of photosynthetic malate in *A. confertifolia* (Fig. 10) may have important regulatory consequences for continued CO_2 assimilation at low temperature. Accumulation of malate in the mesophyll cell cytoplasm could, presumably, inhibit CO_2 fixation via PEP carboxylase (15). Accumulation of malate in bundle sheath cells at low temperature, although less likely, might promote decarboxylation of this substrate by NAD malic enzyme if this is a limiting step at low temperature, and hence promote continued net carbon assimilation. Furthermore, the backing-up of malate in bundle sheath cells or bundle sheath cell mitochondria would not interfere with the movement by diffusion of aspartate from mesophyll cells. At this time, however, further speculation as to the location of the C_4 acid pools and their physiological significance is unprofitable.

The phenomenon of label interchange between C_4 acids during low temperature C_4 photosynthesis in these plants is reminis-

cent of that observed in chilling-sensitive *Sorghum*, an NADP malic enzyme type plant (4). Not surprisingly, the relationship between labeled malate and aspartate is reversed and in *Sorghum* substantial amounts of slowly metabolized aspartate accumulated at low temperature (4). The breakdown in chloroplast membrane functions and the reduction in the photochemical production of NADPH in these plants at low temperature (24, 25, 29) presumably interfere with malate synthesis and metabolism thereby initiating the diversion of ^{14}C to aspartate. The observed changes in radiotracer kinetics of ^{14}C in *Sorghum* at low temperature presumably reflect a total disruption of normal C_4 pathway CO_2 assimilation in this plant.

So far as is known, cold-tolerant C_4 plants do not suffer chloroplast disorganization at low temperature and, consequently, may continue normal photochemical function. Our studies show that C_4 pathway metabolism in these plants shows relatively minor kinetic adjustments during low temperature photosynthesis in these plants. It has been argued that these changes could be such as to promote continued C_4 pathway metabolism in these plants at low temperature—but the arguments rest on a more detailed analysis of C_4 acid distribution and ^{14}C kinetics than is presently available. This tolerance of low temperature may be more a function of chloroplast and membrane stability rather than simply the result of the intricacies of NAD malic enzyme C_4 metabolism.

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LITERATURE CITED

1. Billings WD 1975 Arctic and alpine vegetation: plant adaptations to cold summer climates. *In* JD Ives, RG Barry, eds, Arctic and Alpine Environments. Methuen, London pp 403-443
2. Björkman O 1975 Environmental and biological control of photosynthesis: inaugural address. *In* R Marcelle, ed, Environmental and Biological Control of Photosynthesis. W. Junk, The Hague pp 1-16
3. Black CC 1973 Photosynthetic carbon fixation in relation to net CO_2 uptake. Annu Rev Plant Physiol 24: 253-286
4. Brooking IR, AO Taylor 1973 Plants under climatic stress. V. Chilling and light effects on radiocarbon exchange between photosynthetic intermediates of *Sorghum*. Plant Physiol 52: 180-182
5. Caldwell MM 1972 Adaptability and productivity of species possessing C_3 and C_4 photosynthesis in a cool desert environment. *In* LE Rodin, ed, Ecophysiological Foundation of Ecosystems Productivity in Arid Zone. USSR Academy of Sciences, Leningrad pp 27-29
6. Downton, J, RO Slatyer 1972 Temperature dependence of photosynthesis in cotton. Plant Physiol 50: 518-522
7. Gifford RM 1971 The light response of CO_2 exchange: on the source of differences between C_3 and C_4 species. *In* MD Hatch, CB Osmond, RO Slatyer, eds., Photosynthesis and Photorespiration. Wiley Interscience, New York pp 51-56
8. Gutierrez M, VE Gracen, GE, Edwards 1974 Biochemical and cytological relationships in C_4 plants. Planta 119: 279-280
9. Gutierrez M, R Kanai, SC Huber, SB Ku, GE Edwards 1975 Intracellular localization of carbon metabolism in mesophyll cells of C_4 plants. *In* M Avron, ed, Proceedings of the 3rd International Congress on Photosynthesis. Elsevier, Amsterdam pp 1219-1230
10. Hatch MD 1972 Synthesis of L-malate-4-^{14}C and determination of label in the C-4 carboxyl of L-malate. Anal Biochem 47: 174-183
11. Hatch MD, T Kagawa, S Craig 1975 Subdivision of C_4-pathway species based on differing C_4 acid decarboxylating systems and ultrastructural features. Aust J Plant Physiol 2: 111-128
12. Hatch MD, CB Osmond 1976 Compartmentation and transport in C_4 photosynthesis. *In* CR Stocking, U Heber eds, Encyclopedia of Plant Physiology III. Springer Verlag, Berlin pp 144-184
13. Hilliard JH, SH West 1970 Starch accumulation associated with growth reduction at low temperatures in a tropical plant. Science 168: 494-496
14. Hohorst H-J 1963 L-Malate determination with malic dehydrogenase and DPN. *In* HU Bergmeyer, ed, Methods of Enzymatic Analysis. Verlag Chemic, Academic Press, New York pp 328-332
15. Huber SC, GE Edwards 1975 Inhibition of phosphoenolpyruvate carboxylase from C_4 plants by malate and aspartate. Can J Bot 53: 1925-1933
16. Jarvis PG, RO Slatyer 1966 A controlled-environment chamber for studies of gas exchange by each surface of a leaf. CSIRO Aust. Division of Land Research Technical Paper 29: 1-16
17. Long SP, LD Incoll, HW Woolhouse 1975 C_4 photosynthesis in plants from cool temperate regions, with particular reference to *Spartina townsendii*. Nature 257: 622-624
18. Ludlow MM, GL Wilson 1971 Photosynthesis of tropical pasture plants. I. Illuminance, carbon dioxide concentration, leaf temperature, and leaf-air vapour pressure difference. Aust J Biol Sci 24: 449-470
19. McPherson HG, RO Slatyer 1973 Mechanisms regulating photosynthesis in *Pennisetum typhoides*. Aust J Biol Sci 26: 329-339
20. McWilliam JR, AW Naylor 1967 Interaction of temperature and light in the synthesis of chlorophyll in corn. Plant Physiol 42: 1711-1715
21. Osmond CB, B Harris 1971 Photorespiration during C_4 photosynthesis. Biochim Biophys Acta 234: 270-282
22. Pearcy R, AT Harrison 1974 Comparative photosynthetic and respiratory gas exchange characteristics of *Atriplex lentiformis* (Torr) Wats. in coastal and desert habitats. Ecology 55: 1104-1111
23. Repo E, MD Hatch 1976 Photosynthesis in *Gomphrena celosioides* and its classification amongst C_4 plants. Aust J Plant Physiol 3: 863-876
24. Shneyour A, JK Raison, RM Smillie 1973 The effect of temperature on the rate of photosynthetic electron transport in chloroplasts of chilling-sensitive and chilling-resistant plants. Biochim Biophys Acta 292: 152-161
25. Slack CR, PG Roughman, HCM Bassett 1974 Selective inhibition of mesophyll chloroplast development in some C_4 pathway species by low night temperature. Planta 118: 67-73
26. Slatyer RO 1970 Comparative photosynthesis, growth and transpiration of two species of *Atriplex*. Planta 93: 175-189
27. Stowe LG, JA Teeri 1977 The geographic distribution of C_4 species of the dicotyledonae in relation to climate. Am Naturalist. In press
28. Sutton BG, CB Osmond 1972 Dark CO_2 fixation of CO_2 by Crassulacean plants. Evidence for a single carboxylation step. Plant Physiol 50: 360-365
29. Taylor AO, S Craig 1971 Plants under climatic stress. II. Low temperature, high light effects on chloroplast ultrastructure. Plant Physiol 47: 719-725
30. Taylor AO, JA Rowley 1971 Plants under climatic stress. I. Low temperature, high light effects on photosynthesis. Plant Physiol 47: 713-718
31. Teeri JA, LG Stowe 1976 Climatic patterns and the distribution of C_4 grasses in North America. Oecologia 23: 1-12
32. Vernon LP 1960 Determination of chlorphyll in plant extracts. Anal Chem 32: 1144-1150
33. White RS 1976 Seasonal patterns of photosynthesis and respiration in *Atriplex confertifolia* and *Ceratoides lanata*. PhD dissertation. Utah State Univ