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Biochemical and Cytological Relationships in C_4 Plants

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Summary. C_4 plants can be divided into three groups based on differences in activities of three decarboxylating enzymes: NADP-malic enzyme, NAD-malic enzyme, and phosphopyruvate carboxykinase.

In the *Gramineae* the three C_4 groups are distinguished by anatomical and ultrastructural characteristics of bundle-sheath chloroplasts. NADP-malic enzyme species lack well-developed grana in bundle-sheath chloroplasts (grana reduced) and the bundle-sheath chloroplasts are in the centrifugal position. NAD-malic enzyme species have bundle-sheath chloroplasts in the centripetal position and contain grana. Phosphopyruvate carboxykinase species have bundle-sheath chloroplasts in the centrifugal position and they contain grana. NADP-malic enzyme species of the *Gramineae* have only been found in the subfamilies *Aristidoideae* and *Panicoideae*. With the exception of the genera *Panicum*, and *Urochloa*, NAD-malic enzyme species and phosphopyruvate carboxykinase species have only been found in the subfamily *Eragrostoideae*. C_4 species of the genus *Panicum* are found among all three of the C_4 groups.

The dicotyledonous C_4 species examined fall into two groups: those having high NADP-malic enzyme and those having high NAD-malic enzyme. No phosphopyruvate carboxykinase C_4 species have been found among the dicotyledons. The NADP-malic enzyme C_4 species of the dicotyledons like NADP-malic enzyme species of the *Gramineae* have bundle-sheath chloroplasts with reduced grana but in contrast to NADP-malic enzyme species of the *Gramineae* the bundle-sheath chloroplasts are in the centripetal position. The NAD-malic enzyme species of the dicotyledons like the NAD-malic enzyme species of the *Gramineae* have bundle-sheath chloroplasts in the centripetal position with well developed grana.

The results are discussed in terms of evolutionary and functional diversification of C_4 plants.

Introduction

C_4 plants have a number of common anatomical, physiological and biochemical characteristics, namely, distinct bundle-sheaths consisting of chloroplast-containing cells surrounding the vascular bundles of the leaves (bundle-sheaths), a low CO_2 compensation point, high rates and high temperature optima of photosynthesis, and high activities of phospho-

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pyruvate (PEP) carboxylase, the carboxylating enzyme of the C_4 pathway (see Hatch and Slack, 1970; Black, 1973, for reviews). In comparison to species which lack the C_4-dicarboxylic-acid pathway, C_4 plants also have relatively high $^{13}C/^{12}C$ ratios of leaf dry-matter (Bender, 1968, 1971; Tregunna *et al.*, 1970; Smith and Brown, 1973).

Although C_4 plants have common distinguishing features, it is becoming apparent that there are differences between C_4 species. These differences concern the degree of grana development in the chloroplasts of bundle-sheath cells (Johnson, 1964; Downton, 1970; Black and Mollenhauer, 1971; Laetsch, 1971; Brown and Gracen, 1972); the position of chloroplasts in these cells (Brown, 1960; Brown and Gracen, 1972); and differences in the activities of enzymes related to the pathway of photosynthesis, particularly enzymes thought to function in the decarboxylation of C_4-dicarboxylic acids (Berry *et al.*, 1970; Downton, 1970; Andrews *et al.*, 1971; Edwards *et al.*, 1971; Edwards and Gutierrez, 1972; Huber *et al.*, 1973; Gutierrez *et al.*, 1974; Hatch and Kagawa, 1974). This diversification among C_4 species may reflect differences in the evolution of the C_4 syndrome. In this report, C_4 species of the *Gramineae* and C_4 species among the dicotyledons have been divided into several groups, based on current evidence for cytological and biochemical differences.

Materials and Methods

Plant Culture. Plants were grown in a growth chamber in 16/8-h light-dark cycles with a light/dark temperature of 30/20°. Light was provided by a combination of fluorescent and incandescent lamps giving a quantum flux density, between the wavelengths of 400 and 700 nm, of 40 nE cm^{-2} s^{-1}. For enzyme assays or electron microscopy, leaf samples were taken when the plants were 2–4 weeks of age.

Enzyme Extraction, Enzyme Assays, and Chlorophyll Determination. The enzyme extraction medium contained 0.05 M HEPES (N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid) buffer (pH 7.5), 1 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM dithiothreitol and 2% polyvinylpyrrolidone. About 0.5 g of leaves were ground in a mortar with 3 ml of extraction medium and suspended in a final volume of 10 ml. The homogenate was then passed through a French press at 10000 psi. Without the French-press treatment, the bundle-sheath cells of some species were not broken. The extracts were routinely examined by light microscopy to assure that all leaf cells had been broken. The extraction procedure was carried out at 4° and the crude preparations obtained were used for the enzyme assays.

PEP carboxykinase was assayed in an exchange reaction using $NaH^{14}CO_3$, oxaloacetate and ATP (Edwards *et al.*, 1971); NADP-malic enzyme, spectrophotometrically following NADP reduction as previously described by Kanai and Edwards (1973b); and NAD-malic enzyme, spectrophotometrically following NAD reduction, according to Hatch and Kagawa (1974). The reaction for NAD-malic enzyme was assayed in a double-beam spectrophotometer and the reaction in the sample cuvette initiated by the addition of manganese. A reference cuvette without manganese was included to substract any activity due to NAD-malic dehydrogenase. Evidence for pyruvate formation was routinely checked at the end of the assay by addition of 1.5 units of lactic dehydrogenase and following the reoxidation of NADH. All enzymes were assayed at 30°.

Chlorophyll was determined by the method of Wintermans and De Mots (1965).

Light and Electron Microscopy. For light-microscopy studies leaves were kept in the dark for 36 h before observing the position of the chloroplasts in bundle-sheath cells (centripetal or centrifugal). The chloroplast position was observed with a light microscope in fresh cross-sections of leaves, 50–60 μ thick, cut with an Oxford Model G Vibrotome (Oxford Lab., San Mateo, Calif., U.S.A.), or by observing 1-μ-thick sections of tissue embedded in plastic as for electron-microscopy studies and cut with an ultramicrotome.

For electron microscopy pieces of leaves, 1 mm^2 in size, were fixed in 3% glutaraldehyde in 0.05 M potassium-phosphate buffer, pH 6.8, for 1.5 h; rinsed for 1 h in phosphate buffer, and post-fixed in 2% osmium tetroxide in 0.05 M potassium-phosphate buffer, pH 6.8, for 2 h, then according to Spurr (1969) dehydrated in an acetone series and embedded in a low-viscosity epoxy resin. Fixation and subsequent handling was carried out at room temperature. Electron micrographs of ultrathin sections were made with a Phillips 300 electron microscope.

Results

In Tables 1 and 3, C_4 species are separated into groups based upon three criteria: a) levels of three enzymes, namely, NADP-malic enzyme, NAD-malic enzyme, and PEP carboxykinase, which are now thought to link the C_4 pathway to the reductive pentose-phosphate pathway in C_4 species; b) location of the chloroplasts in the bundle-sheath cells, either centripetal (*i.e.* towards the inner wall) or centrifugal (*i.e.* towards the outer wall); c) degree of grana formation in bundle-sheath-cell chloroplasts: grana either well-developed or reduced (lacking grana or only a few small grana present).

1. Gramineae

Among the C_4 species of the *Gramineae* one group has high NADP-malic enzyme activity, bundle-sheath chloroplasts in the centrifugal position and lacking well developed grana. These NADP-malic enzyme species are found in the subfamilies *Panicoideae* and *Aristidoideae* (Table 1). Magnesium was more effective as a cofactor for NADP-malic enzyme than manganese when compared at a concentration of 20 mM (Table 1). In these species the NAD-malic enzyme and PEP carboxykinase were relatively low in activity with variable activity depending on the species.

A second group among the *Gramineae* has low NADP-malic enzyme, low PEP carboxykinase, but substantial levels of NAD-malic enzyme. The NAD-malic enzyme is manganese dependent, as recently demonstrated by Hatch and Kagawa (1974) for several C_4 species; and in these species the bundle-sheath chloroplasts are in the centripetal position and have well developed grana. These characteristics are found among several species of the genus *Panicum*, subfamily *Panicoideae* and among various species of the subfamily *Eragrostoideae* (Table 1).

Table 1. Classification of C_4 species of the Gramineae into groups based on biochemical and cytological differences[a]

Species	NADP-malic enzyme		NAD-malic enzyme		PEP carboxy-kinase	Bundle-sheath chloroplasts			
						Location		Grana	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}		Centri-fugal	Centri-petal	Re-duced	Well developed
NADP-malic enzyme species									
Aristidoideae									
Aristida purpurea Nutt.[f]	400	532	63	81[e]	0	+[3]			
Panicoideae									
Andropogon gerardii Vitman	705	334	44	25[d]	10	+			
A. scoparius Michx.[h]	445	147	38	96[e]	14	+[5]		+[5]	
Cenchrus pauciflorus Benth	379	23	29	110[e]	56	+			
Digitaria sanguinalis (L.) Scop.	914	844	0	89[c]	82	+[2,4,5]		+[2,5]	
Echinochloa colonum (L.) Link	795	387	123	187[b]	55	+[4]		+[15]	
E. crus-galli (L.) Beauv.	351	206	24	157[e]	61	+			
E. crus-galli var. *frumentacea* (Roxb.) W. F. Wight	1097	810	2	172[b]	225	+			
Euchlaena mexicana Schrad.	953	969	0	92[c]	11	+		+[14]	
Panicum antidotale Retz.	1107	855	0	68[c]	56	+		+	
P. lanipes Mez.	690	538	17	97[e]	19	+			
P. macrophyllum Raddi	927	640	38	0	53	+			
Paspalum notatum Flügge	636	541	0	35[c]	50	+[5]		+[5]	
Pennisetum purpureum Schum.	687	651	21	72[c]	64	+			
Saccharum officinarum L.	1033	1033	0	119[c]	6	+		+[5,15,16]	
Setaria faberii Herrm.	793	405	0	70[e]	1	+			
S. italica (L.) Beauv.	1120	967	0	219[c]	10	+			
S. lutescens (Weigel) Hubb.	463	463	0	64[c]	46	+[5]		+[5]	

S. verticillata (L.) Beauv.	869	828	17	50[c]	0	+			
S. viridis (L.) Beauv.	1608	1056	0	110[c]	6	+[2]		+[2]	
Sorghastrum nutans (L.) Nash.	705	642	0	211[d]	3	+[4]		+[12]	
Sorghum bicolor (L.) Moench	1129	822	7	74[c]	0	+		+[1,5]	
S. sudanense (Piper) Stapf.	1523	838	46	104[e]	<1	+[8]		+[1,9]	
Zea mays L.	1061	810	17	82[c]	25	+[4]		+[5,10,11]	
NAD-malic enzyme species									
Eragrostoideae									
Bouteloua gracilis (H.B.K.) Lag.	11	11	0	120[b, e]	0		+		
Buchloë dactyloides (Nutt.) Engelm	36	36	97	389[e]	0		+		
Chloris distichophylla Lag.	0	0	0	175[e]	0		+		+[5]
Eleusine indica (L.) Gaertn	20	20	0	388[d]	0		+		
Eragrostis cilianensis (All.) Lut.	15	15	0	334[e]	3		+[4]		
E. curvula (Schred.) Nees	15	15	33	176[e]	0		+[4]		+[10]
Leptochloa dubia (H.B.K.) Nees.	75	25	0	178[b]	0		+[4]		+[2]
L. monostachya Roem and Schult.	36	1	12	123[e]	5		+[5]		+[5]
Sporobolus airoides (Torr.) Torr.	0	0	31	169[d]	0		+		
S. cryptandrus (Torr.) A. Gray	8	5	0	134[b, d]	4		+[4]		
Panicoideae									
Panicum bergii Arech	34	29	54	544[e]	0		+[5]		+[5]
P. capillare L.	34	0	0	238[c]	4		+[5]		+[5]
P. coloratum Walt.	108	28	96	406[b]	0	evenly distributed			
P. decompositum R. Br.	33	16	83	314[e]	67		+[5]		+[5]
P. halli Vasey	54	48	70	540[c]	0		+		
P. makarikariense (van Rensb.) Gooss	63	52	52	452[b]	0		+		
P. miliaceum L.	23	0	0	393[c]	0		+[5,8]		+[5,6]
P. turgidum Forsk.	18	24	29	560[b, e]	0		+[5]		+[5]
P. stapfianum Fourc.	60	40	44	527[d]	3		+		

Table 1 (Continued)

Species	NADP-malic enzyme		NAD-malic enzyme		PEP carboxy-kinase	Bundle-sheath chloroplasts			
						Location		Grana	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}		Centri-fugal	Centri-petal	Re-duced	Well developed
PEP carboxykinase species									
Eragrostoideae									
Bouteloua curtipendula (Michx.) Torr.	6	6	12	186[e]	202	+			+[15]
Chloris cucullata Bisch	0	0	0	63[c]	332	+			
C. gayana Kunth	14	12	0	72[c]	465	+			+[17]
Muhlenbergia schreberi J. F. Gmel.	0	0	17	100[c]	364	+[4]			
Sporobolus poiretii (Roem and Schult.) Hitchc.	6	0	0	100[c]	515	+			
Zoysia japonica Steud.	33	16	0	63[d]	156	+			
Panicoideae									
Urochloa panicoides Beauv.	46	15	53	177[d]	348	+			
Panicum maximum Jacq.	14	7	0	142[c]	264	+[4g]			+[5]
P. molle Michx.	4	4	21	196[d]	310	+			
P. texanum Buckl.	34	0	0	152[c]	225	+[4g]			+[5]
C_3-Plants									
Festucoideae									
Agropyron repens (L.) Beauv.	18	0	0	53[b]	0				
Hordeum vulgare L.	25	21	27	128[b]	0				+
Stipa viridula Trin.	14	14	0	78[e]	0				
Triticum aestivum L.	13	6	0	40[c]	3				

Panicoideae						
Dichanthelium clandestinum (L.) Gould	0	0	0	130[b]	0	
Panicum bisulcatum Thumb.	5	5	0	148[b]	4	+
P. tricanthum Nees.	0	0	0	119[d]	0	

[a] Data expressed as μmol mg-chlorophyll h^{-1}.

[b–e] The assay of NAD-malic enzyme with Mn^{2+} was run with the following additions: [b] none, [c] 8 mM $(NH_4)_2SO_4$, [d] 75 μM CoA, [e] 25 μM acetyl CoA according to Hatch and Kagawa (1974). The condition giving the maximum rate with each species is reported and the conditions giving maximum activity with Mn^{2+} were then used to assay NAD-malic enzyme with Mg^{2+}.

[f] Species of the genus *Aristida* examined show a double bundle-sheath layer, with the inner layer having agranal, centrifugal chloroplasts (Johnson, 1964; Johnson and Brown, 1973).

[g] Essentially centrifugal but a few plastids are on centripetal side.

[h] *Andropogon scoparius* as given in ref. 5 is now referred to as *Schizachyrium scoparium* Nash.

References: 1 Bisalputra *et al.* (1969). — 2 Black and Mollenhauer (1971). — 3 Brown (1958). — 4 Brown (1960). — 5 Brown and Gracen (1972). — 6 Downton (1970). — 7 Downton (1971). — 8 Downton *et al.* (1970). — 9 Fredrick and Newcomb (1971). — 10 Gracen *et al.* (1972). — 11 Hodge *et al.* (1955). — 12 Johnson (1964). — 13 Laetsch (1968). — 14 Laetsch (1969). — 15 Laetsch (1971). — 16 Laetsch *et al.* (1966). — 17 Woo *et al.* (1971).

The third group of C_4 species of the *Gramineae* has high PEP carboxykinase activity and possesses bundle-sheath chloroplasts which are predominantly centrifugal and have well-developed grana. PEP carboxykinase species have been found in the *Eragrostoideae* subfamily and some species in the genera *Panicum* and *Urochloa* of the *Panicoideae* (Table 1). This group is characterized biochemically by the presence of high PEP carboxykinase and not by the exclusion of other C_4-acid decarboxylating enzymes since some species having high PEP carboxykinase also have substantial NAD-malic enzyme (Table 1).

All the C_4 species with high NADP-malic enzyme are found in the subfamily *Panicoideae* while species of the subfamily *Eragrostoideae* have either high PEP carboxykinase or high NAD-malic enzyme. Species of *Panicum* are found among all three groups of C_4 *Gramineae*, and this genus also contains C_3 plants.

As expected, the level of PEP carboxykinase, NADP-malic enzyme, and NAD-malic enzyme activity in C_3 grasses was low as compared to the C_4 grasses. However, in most species, including the C_3 grasses, there was substantial activity of manganese-dependent NAD-malic enzyme although the highest activities were found in the C_4 grasses having low NADP-malic enzyme and low PEP carboxykinase (Table 1). Table 2 shows that manganese-dependent NAD-malic enzyme is localized in the bundle-sheath cells of various C_4 grasses. There is substantial activity of the enzyme in bundle-sheath cells of species representing all three C_4 groups of the *Gramineae*.

Table 2. Distribution of NAD-malic enzyme between mesophyll and bundle-sheath cells of various C_4 grasses[a]

Group	Species	Mesophyll-protoplast extracts		Bundle-sheath cell extracts	
		Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}
NADP-malic enzyme species	*Pennisetum purpureum*	0	10	69	208
	Zea mays	0	0	37	126
NAD-malic enzyme species	*Eleusine indica*	44	11	46	554
	Panicum miliaceum	0	38	62	413
PEP carboxykinase species	*Panicum texanum*	23	62	43	263
	Urochloa panicoides	0	7	38	308

[a] Data expressed as μmol mg^{-1} chlorophyll h^{-1}. See Table 1 for optimum conditions with each species. Isolations were made according to Kanai and Edwards (1973b), Gutierrez *et al.*, (1974).

The characteristics of the bundle-sheath chloroplasts of the three group of C_4 grasses can be illustrated with *Panicum antidotale* (NADP-malic enzyme species), *Panicum miliaceum* (NAD-malic enzyme species) and *Panicum maximum* (PEP carboxykinase species). Fig. 1A shows light micrographs of *P. antidotale* with centrifugal bundle-sheath chloroplasts; Fig. 1B, of *P. miliaceum* with bundle-sheath chloroplasts in the centripetal position; and Fig. 1C, of *P. maximum* with most of the bundle-sheath chloroplasts again in the centrifugal position. As the bundle-sheath chloroplasts become filled with starch, their volume increases and they tend to fill the entire bundle-sheath cells, thus obscuring the centrifugal or centripetal arrangement. However, a few bundle-sheath chloroplasts of *P. maximum* and *P. texanum* are often seen on the centripetal cell wall even in cells devoid of starch. Electron micrographs of bundle-sheath chloroplasts of *P. antidotale* show lack of a well-developed grana system (Fig. 2A); only a few rudimentary grana were usually observed. In contrast, *P. miliaceum* which has a centripetal chloroplast arrangement, and *P. maximum* which has essentially a centrifugal chloroplast arrangement in the bundle-sheath cells have bundle-sheath chloroplasts with well-developed grana (Fig. 2B and C).

2. Dicotyledonous Species

Among the dicotyledons examined two groups of C_4 species have been found. One group, represented by species of *Kochia*, *Euphorbia*, *Portulaca* and *Froelichia*, has high NADP-malic enzyme activity, and bundle-sheath chloroplasts in the centripetal position lacking well-developed grana. Magnesium is more effective than manganese as cofactor for the NADP-malic enzyme, particularly in *Froelichia gracilis* (Table 3).

The other group of dicotyledons, represented by *Amaranthus* species and *Portulaca oleracea*, is characterized by high manganese-dependent NAD-malic enzyme activity, and bundle-sheath chloroplasts in the centripetal position with well-developed grana. As expected, the levels of the three decarboxylating enzymes in the C_3 dicotyledons *Nicotiana tabacum* and *Phaseolus vulgaris* were low (Table 3).

Fig. 1A—C. Cross sections of leaves of *Panicum antidotale* (A), *P. miliaceum* (B) and *P. maximum* (C); × 400. The chloroplasts (*C*) in the bundle-sheath cells surrounding the vascular bundle (*VB*) demonstrate characteristic patterns of distribution. Location of chloroplasts on the bundle-sheath cell wall nearest the vascular bundle, centripetal arrangement, is seen in B; location of chloroplasts on the cell wall opposite the vascular bundle, centrifugal arrangement, is seen in (A) and (C)

Fig. 2A—C. Bundle-sheath chloroplasts of *Panicum antidotale* (A), *P. miliaceum* (C) and *P. maximum* (C). A well-developed pattern of granal stacks (*G*) is apparent in (B) and (C); the number and size of grana are reduced in A. (A) and (B) × 18, 275; (C) × 20,150

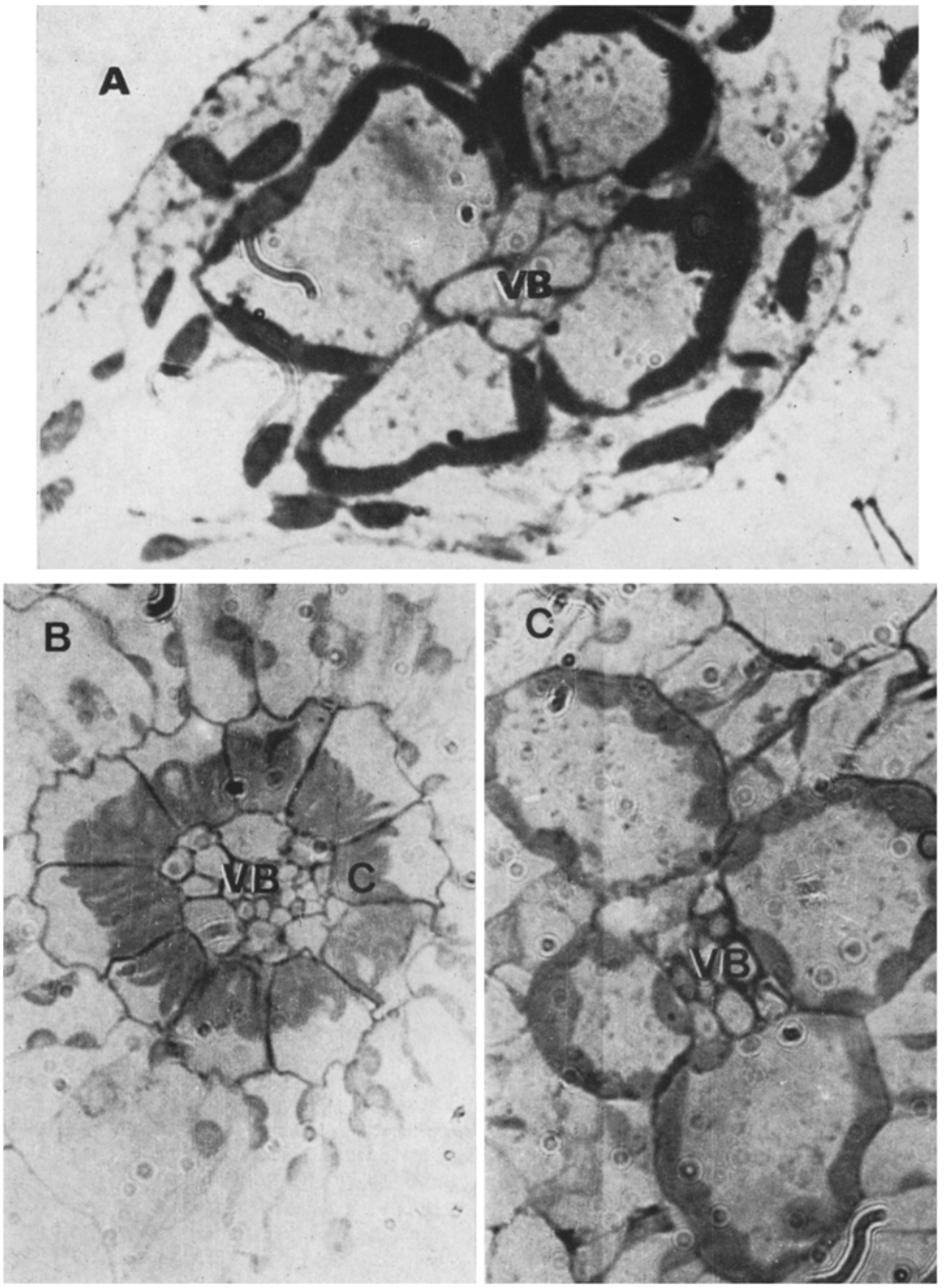

Fig. 1 (for legend see p. 287)

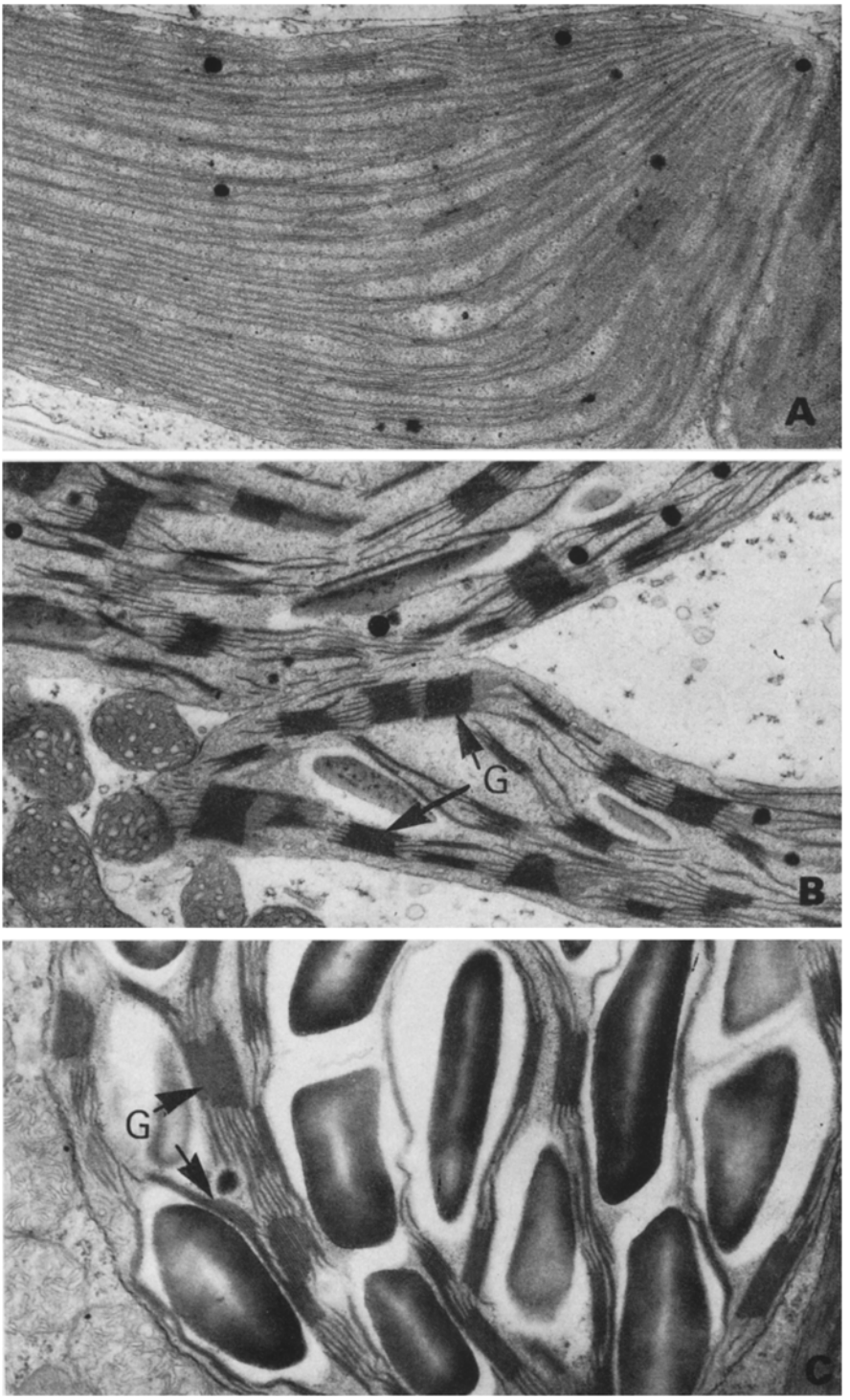

Fig. 2 (for legend see p. 287)

Table 3. Classification of C_4 species of some dicotyledonous plants into groups based on biochemical and cytological differences[a]

Species	NADP-malic enzyme		NAD-malic enzyme		PEP carboxykinase	Bundle-sheath chloroplasts			
						Location		Grana	
	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}		Centrifugal	Centripetal	Reduced	Well developed
NADP-malic enzyme species									
Amaranthaceae									
Froelichia gracilis (Hook) Mog.	781	57	11	59[e]	<1		+	+[15]	
Gomphrena globosa L.	465	392	0	267[c]	5		+[7]	+[7]	
Chenopodiaceae									
Kochia childsii L.	529	136	41	107[c]	0		+		
K. scoparia (L.) Schrad	745	549	27	113[c]	0		+		
Salsola kali L.	318	94	42	84[d, e]	0		+		
Euphorbiaceae									
Euphorbia maculata L.	1520	1092	35	176[c]	27		+	+[15]	
E. supina Raf.	420	302	0	60[c]	0		+		
Portulacaceae									
Portulaca grandiflora Hook	345	218	48	144[b]	0		+		
NAD-malic enzyme species									
Amaranthaceae									
Amaranthus edulis Speg.	58	42	68	257[e]	0		+[13]		+[13]
A. hybridus L.	40	34	0	380[c]	6		+		
A. retroflexus L.	66	37	0	600[c]	0		+[2]		+[2]
A. tricolor L.	0	0	0	408[d, e]	10		+		
Portulacaceae									
Portulaca olereacea L.	60	39	0	560[c]	40		+		+[14]
C_3-Plants									
Leguminosae									
Phaseolus vulgaris L.	7	0	7	0	0				
Solanaceae									
Nicotiana tabacum L.	9	25	7	31[d]	0				

[a–e] See Table 1.
[2,7,13,14,15] See references Table 1.

Discussion

This report brings together evidence, both from the literature and from our own investigations, which allows the classification of C_4 species into groups based on cytological and biochemical information. Brown in 1960 recognized that species of the subfamily *Panicoideae* of the *Gramineae* have bundle-sheath chloroplasts in the centrifugal position while the subfamily *Eragrostoideae* has both species with bundle-sheath chloroplasts in the centripetal and the centrifugal position. More recent investigations have shown that the genus *Panicum*, subfamily *Panicoideae*, a very diversified genus, has centripetal bundle-sheath chloroplasts in some species and centrifugal ones in others (Downton, 1971; Brown and Gracen, 1972; Table 1). Johnson (1964) and Johnson and Brown (1973) examined 32 species in 16 tribes of grasses and found that in the *Panicoideae* the chloroplasts of the bundle-sheath cells tended to have reduced grana while in the *Eragrostoideae* they contained well-developed grana. Our studies show that the genus *Panicum* contains species with bundle-sheath chloroplasts which tend to be agranal as well as species which have bundle-sheath chloroplasts with well-developed grana (Table 1, Fig. 2). Thus far this is the only genus found among the *Panicoideae* or *Eragrostoideae* which contains both species with largely agranal and species with granal bundle-sheath chloroplasts.

The association of C_4 photosynthesis with the Kranz-type leaf anatomy by Downton and Tregunna (1968) and Laetsch (1968) eventually led to a recognition of biochemical and chloroplast-ultrastructural differences within C_4 species. *Saccharum officinarum* and *Sorghum bicolor* which have agranal bundle-sheath chloroplasts, and *Zea mays* and *Digitaria sanguinalis* which have a few rudimentary grana in these chloroplasts have high levels of NADP-malic enzyme in the bundle-sheath cells (Berry *et al.*, 1970; Downton, 1970; Edwards and Black, 1971; Kanai and Edwards, 1973b, Gutierrez *et al.*, 1974). All of the species of the *Panicoideae* which we have found to possess high levels of NADP-malic enzyme have bundle-sheath chloroplasts which are in the centrifugal position and are either agranal or lacking well-developed grana (Table 1).

Recently Hatch and Kagawa (1974) reported high NAD-malic enzyme in certain C_4 species including both monocotyledons and dicotyledons. Species among the *Gramineae* which have bundle-sheath chloroplasts in the centripetal position with well-developed grana have low levels of NADP-malic enzyme, but substantial levels of NAD-malic enzyme (Table 1). With the exception of the genus *Panicum*, these characteristics have only been found in the subfamily *Eragrostoideae*.

In 1971, Edwards *et al.* found high PEP carboxykinase in the C_4 species *Panicum maximum*, *Panicum texanum* and *Sporobolus poiretii*. The PEP carboxykinase species identified have bundle-sheath chloro-

plasts in the centrifugal position and contain grana (Table 1). These species also contain some NAD-malic enzyme. It should be noted that NAD-malic enzyme activity is found in all groups with substantial activity in some PEP carboxykinase and NADP-malic enzyme species. Therefore it is conceivable that there is an additional carboxyl-donating enzyme in some grasses currently designated NAD-malic enzyme species.

Studies of leaf anatomy, CO_2 compensation point, and $^{13}C/^{12}C$ ratios of the leaf have lead to the classification of species of the *Eragrostoideae* and most species of the *Panicoideae* as C_4 plants; and species of the subfamilies *Festucoideae*, *Arundinoideae* and *Oryzoideae* as C_3 plants (Downton and Tregunna, 1968; Smith and Brown, 1973). Three groups of C_4 species can be recognized based on the criteria of the present study (Table 1). Brown and Smith (1972) suggested that the evolution of the Kranz anatomy occurred several times in the *Gramineae* and that the diversity of the genus *Panicum* may provide clues to the evolution of the grass family.

A scheme showing the diversification of C_4 plants among the subfamilies *Aristidoideae, Panicoideae* and *Eragrostoideae* based on biochemical and cytological characteristics is shown in Fig. 3. In the *Panicoideae* genus *Panicum*, all three groups of C_4 species are found. NADP-malic enzyme species are found only among the *Panicoideae* and *Aristidoideae* while in the *Eragrostoideae* both PEP carboxykinase and NAD-malic enzyme species are found. Genera of the *Eragrostoideae* having both NAD-malic enzyme and PEP carboxykinase species are *Bouteloua*, *Chloris* and *Sporobolus*. The classification presented is tentative and may be expanded or modified as other C_4 species are examined. Although most species of the *Gramineae* which we have examined fit into the groups of Fig. 3, we have already found some exceptions based either on biochemical or cytological deviations. These exceptions, which require further study, include *Panicum virgatum* (bundle-sheath chloroplasts evenly distributed, decarboxylating enzymes low); *P. laevifolium*, *P. dichotomiflorum* and *Muhlenbergia lindheimeri* (bundle-sheath chloroplasts centrifugal, decarboxylating enzymes low).

Although the Kranz-type leaf anatomy seems most prevalent among the monocotyledons, cytological and biochemical variations can be found among several C_4 dicotyledons, even though no C_4 dicotyledon has yet been found which has high PEP carboxykinase activity. NADP-malic enzyme species of dicotyledons have been found in the genera *Kochia*, *Euphorbia*, *Froelichia*, *Salsola* and *Portulaca*; while NAD-malic enzyme species of the dicotyledons have been found in the genera *Amaranthus* and *Portulaca*. *Atriplex* species which have granal bundle-sheath chloroplasts in the centripetal position (Laetsch, 1968; Osmond *et al.*, 1969) also have high NAD-malic enzyme (Hatch and Kagawa, 1974).

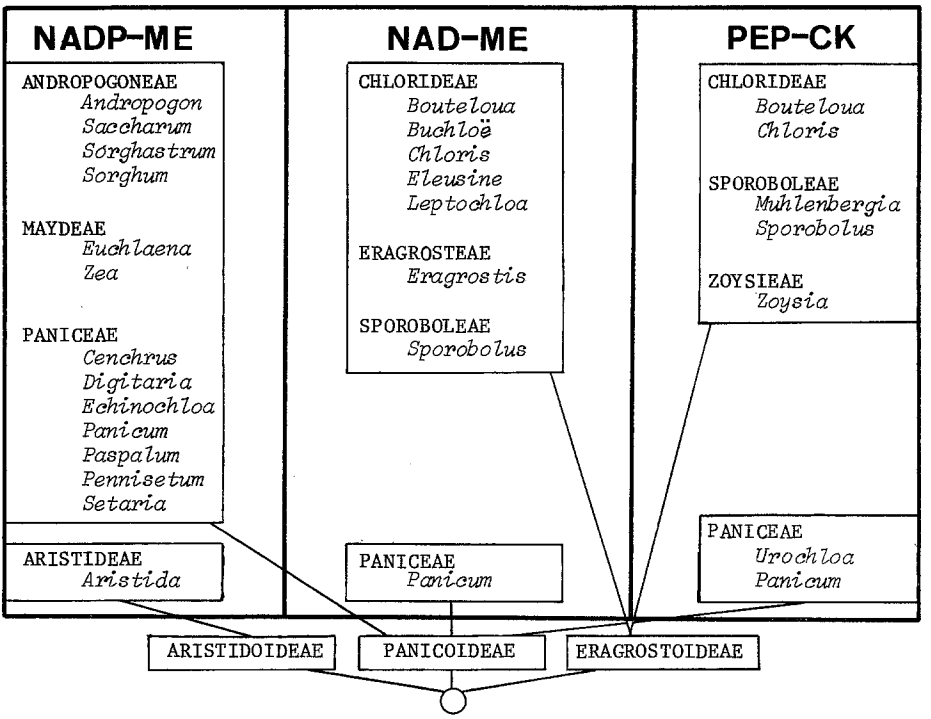

Fig. 3. Phylogenetic scheme of C_4 groups in the *Gramineae* based on biochemical and cytological examinations of species of the subfamilies *Aristidoideae*, *Eragrostoideae* and *Panicoideae*

The significance of the cytological and biochemical correlations among C_4 monocytoledons and dicotyledons is of interest from both a functional and evolutionary standpoint. In 1961, Brown suggested "... the physiological and anatomical specializations of the parenchyma sheath are interrelated and are important in certain fundamental life processes". Recent studies indicate that species of the *Panicoideae* which have high NADP-malic enzyme activity and bundle-sheath chloroplasts without grana, or with reduced ones, either may lack Hill-reaction activity, examples being *Sorghum bicolor* and *Saccharum officinarum*, or have little Hill-reaction activity, examples being *Zea mays*, *Digitaria sanguinalis* and *Setaria lutescens* (Downton *et al.*, 1970; Anderson *et al.*, 1971; Mayne *et al.*, 1971; Ku *et al.*, 1974a; Osmond, 1974; Mayne *et al.*, in press). This evidence suggests a correlation between degree of grana development and level of noncyclic electron transport from the Hill reaction in the bundle-sheath chloroplasts. It is proposed in these species, *i.e.*, species

having NADP-malic enzyme that malate, initially formed in mesophyll cells by the C_4 pathway, is transported to the bundle-sheath cells and decarboxylated by NADP-malic enzyme to form NADPH with the production of CO_2. The NADPH generated by malic enzyme would provide part of the reducing power for fixation of the CO_2 in the bundle-sheath cells through the reductive pentose-phosphate pathway (Downton, 1970; Edwards and Black, 1971; Hatch, 1971; Huber *et al.*, 1973). In NADP-malic enzyme species most, and in some species all of the reductive power for reducing the products of the reductive pentose-phosphate pathway may be generated by the mesophyll chloroplasts.

NAD-malic enzyme species of the *Gramineae* are proposed to transport aspartate, the product of C_4 photosynthesis in the mesophyll cells, from the latter to the bundle-sheath cells. In the bundle-sheath cells, aspartate would be converted to oxaloacetate (OAA), OAA to malate, and malate to pyruvate and CO_2 by aspartate transaminase, malate dehydrogenase, and NAD-malic enzyme, respectively (Hatch and Kagawa, 1974). In PEP carboxykinase species, aspartate would be transported from the mesophyll cells to the bundle-sheath cells and converted to OAA by aspartate transaminase. OAA would be converted to PEP and CO_2 by PEP carboxykinase (Edwards *et al.*, 1971, 1974). In either case, these reactions would produce CO_2 but would not result in net synthesis of reducing power in the bundle-sheath cells. Therefore, the grana-containing bundle-sheath chloroplasts which are characteristic for both NAD-malic enzyme and PEP carboxykinase species may supply through non-cyclic electron transport the reducing power required for fixation of the CO_2 through the reductive pentose-phosphate pathway. Thus, in contrast to NADP-malic enzyme species the bundle-sheath chloroplasts of NAD-malic enzyme and PEP carboxykinase species of the *Gramineae* may have a primary role in producing reductive power for the reductive pentose phosphate pathway. This interpretation is supported by the finding that a large part of photosystem II is in bundle-sheath cells of NAD-malic enzyme and PEP carboxykinase species in comparison to NADP-malic enzyme species (Ku *et al.*, 1974a; Mayne *et al.*, in press).

C_4 species of the various groups of the *Gramineae* thus appear to differ in cytology, the photochemical capacity of the chloroplasts of the bundle-sheath cells, and the mechanism of carboxyl transfer from the C_4 acids to the reductive pentose-phosphate pathway. A common feature of the three C_4 groups of the *Gramineae*, based on the analysis of several species, seems to be the localization of the C_4 pathway in the mesophyll cells and of the carboxylation phase of the reductive pentose phosphate pathway in the bundle-sheath cells (Gutierrez *et al*, 1974; Ku *et al.*, 1974b). There is also evidence that the decarboxylating enzymes are localized in the

bundle-sheath cells (Edwards and Black, 1971; Chen *et al.*, 1973; Huber *et al.*, 1973; Kanai and Edwards, 1973a, b; Gutierrez *et al.*, 1974; Hatch and Kagawa, 1974; Table 2). In the case of the C_4 dicotyledons which have either high NADP-malic enzyme or high NAD-malic enzyme, information on the photochemical capacity and distribution of photosynthetic enzymes between the two cell types is not yet sufficient to establish correlations between their cytological and biochemical characteristics.

The position of chloroplasts in the bundle-sheath cells of the *Gramineae* may have evolutionary relevance. If the loss of grana in the bundle-sheath chloroplasts is the most specialized and evolutionary advanced situation in C_4 grasses, a possible evolutionary pattern would be from NAD-malic enzyme species with centripetal chloroplasts to PEP carboxykinase species with predominantly centrifugal chloroplasts (some species retaining NAD-malic enzyme) to NADP-malic enzyme species with centrifugal chloroplasts and varying degrees of grana reduction in bundle-sheath chloroplasts.

The functional significance of the position of bundle-sheath chloroplasts in various C_4 species is not known. As noted by Hatch and Slack (1970) the centrifugal arrangement of these chloroplasts appears to be ideal for the exchange of photosynthetic intermediates between these chloroplasts and those of the mesophyll cells. Species of monocotyledons and dicotyledons which have high NADP-malic enzyme may have a similar pathway of photosynthesis. However, the monocotyledons with high NADP-malic enzyme have centrifugal bundle-sheath chloroplasts while the dicotyledons with high NADP-malic enzyme have centripetal ones. Further information on the pathway of photosynthesis in the two cell types, including the intracellular location of the enzymes for decarboxylation of C_4 acids, may be helpful for relating bundle-sheath chloroplast position to chloroplast function.

Although most species of C_4 monocotyledons and dicotyledons fit into distinct groups based on the levels of proposed decarboxylating enzymes and cytology, a particular C_4 species may utilize more than one mechanism for carboxyl donation to the reductive pentose-phosphate pathway. It was previously suggested that *Gomphrena globosa* may be an intermediate-type C_4 species (Andrews *et al.*, 1971). The levels of NADP-malic enzyme and NAD-malic enzyme (Table 3) support this suggestion. Species like *Echinochola crus-galli* var. *frumentacea* (Table 1) may likewise utilize PEP carboxykinase as well as NADP-malic enzyme in carboxyl transfer to the reductive pentose-phosphate pathway; and some NADP-malic enzyme and PEP carboxykinase species may also use NAD-malic enzyme in this transfer (Tables 1, 2).

It seems appropriate to distinguish C_4 species generally on the basis of the primary mechanism of C_4 acid decarboxylation, *i.e.*, NADP-malic

Table 4. Average activities of decarboxylating enzymes in various groups of plants[a]

Group	Number species	NADP-malic enzyme		NAD-malic enzyme		PEP carboxy-kinase
		Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	
Monocotyledons						
NADP-malic enzyme species	24	850	639	20	99	36
NAD-malic enzyme species	20	32	18	30	308	4
PEP carboxykinase species	10	15	6	10	125	318
C_3 species	7	10	6	4	99	1
Dicotyledons						
NADP-malic enzyme species	8	640	355	25	126	4
NAD-malic enzyme species	5	45	30	14	441	11
C_3 species	2	8	12	7	15	0

[a] Averages are from data in Tables 1 and 3. Activity is expressed as μmol mg^{-1} chlorophyll h^{-1}.

enzyme species, NAD-malic enzyme species, PEP carboxykinase species. This classification, although appropriate for most species studied, should not exclude the possibility that a given C_4 species may use more than one C_4 acid decarboxylation enzyme for carboxyl donation to the reductive pentose-phosphate pathway. In Table 4, averages of the activities of NADP-malic enzyme, NAD-malic enzyme and PEP carboxykinase are given for the three groups of monocotyledons and the two groups of dicotyledons. Of all the species studied, in NADP-malic enzyme species *ca.* 85% of the decarboxylating activity is in NADP-malic enzyme (Mg^{2+} dependent), in NAD-malic enzyme species *ca.* 90% of the decarboxylating activity is in NAD-malic enzyme (Mn^{2+} dependent) and in PEP carboxykinase species *ca.* 70% of the decarboxylating activity is in PEP carboxykinase. Different C_4 species may accomplish the same end photosynthetically, *i.e.*, supply the reductive pentose-phosphate pathway with a high concentration of CO_2, through different mechanisms but direct evidence for the relative role of decarboxylating enzymes in carboxyl donation from C_4 acids to the reductive pentose-phosphate pathway in different C_4 species is so far not available.

For future reference in classifying a C_4 species the description could include the following biochemical and cytological characteristics. Although the classification may be extended, the major groups thus far are:

1. Monocotyledons
   2. *NADP-malic enzyme species*
      3. Bundle-sheath chloroplasts with reduced grana (R)
         4. Bundle-sheath chloroplasts centrifugal (f) *i.e.* Rf *Saccharum officinarum*
   2. *NAD-malic enzyme species*
      3. Bundle-sheath chloroplasts with well developed grana (D)
         4. Bundle-sheath chloroplasts centripetal (p) *i.e.* Dp *Panicum miliaceum*
   2. *PEP carboxykinase species*
      3. Bundle-sheath chloroplasts with well developed grana (D)
         4. Bundle-sheath chloroplasts centrifugal (f) *i.e.* Df *Panicum maximum*
1. Dicotyledons
   2. *NADP-malic enzyme species*
      3. Bundle-sheath chloroplasts with reduced grana (R)
         4. Bundle-sheath chloroplasts centripetal (p) *i.e.* Rp *Froelichia gracilis*
   2. *NAD-malic enzyme species*
      3. Bundle-sheath chloroplasts with well developed grana (D)
         4. Bundle-sheath chloroplasts centripetal (p) *i.e.* Dp *Amaranthus retroflexus*

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Addendum. Dr. W. V. Brown, Austin, Texas has noted that in the Gramineae NADP-malic enzyme species have Kranz sheath developing from mestome sheath while PEP carboxykinase and NAD-malic enzyme species have Kranz sheath coming from ground parenchyma. This anatomical distinction in the grasses may also be used as a basis for classification (personal communication).