Phytochemistry, Vol. 25, No. 7, pp. 1537–1543, 1986.
Printed in Great Britain.

0031-9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

SURVEY OF PYRUVATE, PHOSPHATE DIKINASE ACTIVITY OF PLANTS IN RELATION TO THE C_3, C_4 AND CAM MECHANISMS OF CO_2 ASSIMILATION

COLIN G. HOCKING* and JOHN W. ANDERSON

Botany Department, La Trobe University, Bundoora, Victoria 3083, Australia

(*Revised received* 25 *April* 1985)

Key Word Index—Amaranthaceae; Crassulaceae; Gramineae; Hydrocharitaceae; Orchidaceae; Solanaceae; Xanthorrhoeaceae; C_4 plants; C_3 plants; CAM plants; pyruvate, phosphate dikinase; phosphoenolpyruvate metabolism.

Abstract—The pyruvate, phosphate dikinase activity (PPD, EC 2.7.9.1) associated with crude extracts of leaf tissue of some C_3 and C_4 plants was determined by phosphoenolpyruvate plus PPi-dependent phosphorylation of AMP. The PPD activity of all C_4 plants examined was > 15 nmol/mg protein/min. Several factors contributed to the underestimation of PPD activity in crude extracts of at least some species. Significant PPD activity (> 0.15 nmol/mg protein/min) was not detected in the majority of C_3 species but several C_3 species and the two CAM species studied exhibited activity in the range 0.4–4 nmol/mg protein/min while the C_3 species *Avena sativa* showed activity up to 8 nmol/mg protein/min. The oat leaf enzyme was partially purified; it exhibited properties similar to those of partially purified PPD from maize. Leaf extracts of the orchids *Cymbidium canaliculatum* and *C. madidum* contained high levels of PPD activity similar to the majority of C_4 plants. PPD activity has also been shown in other previously unstudied species.

INTRODUCTION

The synthesis of phosphoenolpyruvate (PEP) through the action of pyruvate, phosphate dikinase (PPD, EC 2.7.9.1) is regarded as an essential adjunct to the C_4 mechanism of CO_2 incorporation in most C_4 plants [1]. Since PPD is reportedly absent or occurs in very low activity in the leaves of C_3 plants [2–7], this raises the question whether the activity of this enzyme might afford a means of determining the mechanism of CO_2 assimilation in species in which this has not been established. This, however, necessitates a simple, reliable and specific method for the determination of PPD activity in crude leaf extracts of C_3, C_4 and CAM plants. Methods based either directly or indirectly on the production of pyruvate from PEP are subject to high endogenous non-specific activity and/or interference from competing enzymes [3, 4]. These processes could mask PPD activity in species in which the activity of the enzyme is very low. Perhaps significantly, PPD has not been reported in the leaf tissue of C_3 plants when this method has been employed [2, 4].

Recently, we reported that the PEP plus PPi-dependent phosphorylation of AMP, previously described for purified PPD [8], provides a simple, sensitive and reliable method for determining PPD activity in crude extracts of maize [9]. In this paper we report a survey of various C_3, C_4 and CAM plants for PPD activity using the phosphorylation assay. Low level PPD activity was detected in the leaves of several C_3 species and some properties of the enzyme from *Avena sativa* are described.

RESULTS

Effect of post-harvest storage on PPD activity of maize leaf tissue

Experiments were undertaken to determine conditions for transporting harvested leaf material to the laboratory without loss of PPD activity. The treatments described below were always followed by a period of 2 hr illumination at room temperature immediately prior to extraction. Relative to freshly detached tissue, the extractable PPD activity of illuminated detached leaves (maintained with their leaf bases in water) decreased by *ca* 35% over a period of 4–8 hr. The activity of detached leaves stored in the dark in a plastic bag at 30° for 6–8 hr decreased by *ca* 55% while the activity of material stored in the same manner at 0° decreased by *ca* 45%. Accordingly freshly harvested tissue was used in the ensuing studies whenever possible but when this was impractical, harvested material was stored on ice during transit to the laboratory and pre-illuminated for 2 hr immediately prior to extraction.

Effect of light on the extractable PPD activity of leaf tissue of maize and Amaranthus edulis

The PPD of C_4 plants as determined by methods which monitor pyruvate formation is subject to reversible short term light activation [10, 11]. Accordingly, the activity catalysing PEP plus PPi-dependent phosphorylation of

*Present address: Applied Science Department, Holmsglen College of Technical and Further Education, 900 Dandenong Road, Caulfield E., Victoria 3145, Australia.

AMP was examined with respect to illumination in maize. Relative to illuminated plants, the activity of plants placed in the dark decreased rapidly (65% and 73% decrease after 20 and 65 min respectively). Upon re-illumination the activity returned to 82% of the initial activity after 100 min. These observations confirm the validity of PEP plus PPi-dependent phosphorylation of AMP as a measure of PPD activity. They also show that this characteristic could be used for documenting PPD activity in species which have not been investigated previously.

To examine the long term effect of light on PPD activity, plants of *Zea mays* and *Amaranthus edulis* were raised under various light intensities. Expressed per unit of Chl, the PPD activity of maize and *A. edulis* increased with irradiance up to *ca* 300 and 800 $\mu E/m^2/sec$ respectively; at these values the PPD activity was *ca* 4.5 to 5-fold greater than the activity of plants grown at 70 $\mu E/m^2/sec$ (Fig. 1). The highest level of PPD activity in *A. edulis* was only 40% of maize and only 33% when expressed per unit protein. Although extraction differences between the two species cannot be discounted as the cause of the differing light respone curves, similar differences have been reported for maize and *A. palmeri* with respect to the effect of irradiance on CO_2 assimilation [12] suggesting that light regulation of PPD activity during growth could influence the rate of CO_2 assimilation in C_4 plants. The results confirm the earlier findings of Yamomoto [11] and also show that plants should be grown at the highest possible irradiance for maximum PPD activity. NH_4Cl (50 mM) invariably enhanced the PPD activity of crude extracts of maize and *A. edulis ca* 2.5 to 3-fold, regardless of the irradiance of the plants during growth. NH_4^+ activation [8] and long term light induction of PPD activity must therefore involve separate mechanisms.

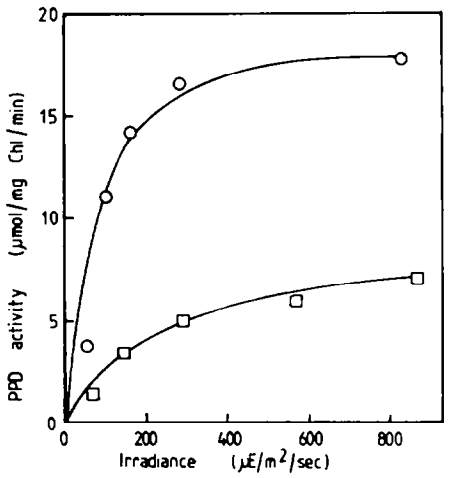

Fig. 1. Effect of irradiance during growth on the PPD activity of leaves of *Zea mays* (○) and *Amaranthus edulis* (□). Plants were grown under fluorescent lights in a growth cabinet on a 14 hr/10 hr light/dark cycle at 30°/24°. Irradiance was varied with neutral density filters and PPD activity determined after 8 weeks growth. All plants were irradiated in full sun light (2100 $\mu E/m^2/sec$) for 2 hr immediately prior to extraction and assay.

Survey of plants for PPD activity

Tables 1 and 2 list the PPD activity of crude extracts prepared from pre-illuminated leaves of various species as determined in standard assays containing 50 mM NH_4Cl and corrected for PPi incorporation in the absence of AMP and PEP. In Table 1, the activity in the absence of NH_4Cl (corrected for activity in the absence of PEP and AMP) is also shown. The data show that all of the species previously classified as C_4 by other criteria exhibit PPD activity in excess of 15 nmol/mg protein/min in the presence of NH_4Cl. Conversely the PPD activity of all species previously classified as C_3 was less than 4 nmol/mg protein/min in the presence of NH_4Cl. Although many of the C_3 species exhibited insignificant or undetectable PPD activity (Table 2), several C_3 plants (e.g. *Avena sativa*) and plants assumed to be C_3 plants because of their phylogeny (e.g. *Bromus mollis*) contained a significantly low level of PPD activity (0.4–4 nmol/mg protein/min) as determined by the PPi incorporation technique (Table 1). In other experiments, some extracts of *Avena sativa* exhibited PPD activity up to 8 nmol/mg protein/min. Both of the CAM plants investigated (*Kalanchoe daigremontiana* and *Aloe arborescens*) also showed PPD activity in the range 0.4–4 nmol/mg protein/min and therefore could not be distinguished from the more active of the C_3 plants by their PPD activity. Several orchids showed significant low level PPD activity (0.4–5 nmol/mg protein/min) and three species exhibited activity > 10 nmol/mg protein/min. Two of these, *Cymbidium canaliculatum* and *C. madidum*, had PPD activities which were surpassed only by four of the most active C_4 plants.

Evidence for endogenous PPD inhibitors in plants

The rates of PPD activity in many of the C_4 species examined (e.g. 9.7 and 4.1 μmol/mg Chl/hr in *Paspalum dilatatum* and *Themeda australis* respectively) were inadequate to explain the rates of CO_2 assimilation typical of C_4 plants. To test for the possibility of an endogenous PPD inhibitor in extracts of species showing relatively low PPD activity the effect of extracts of *T. australis* and *P. dilatatum* on the activity of maize leaf PPD (which exhibited very high activity) was examined. The activity of extracts of *Z. mays* and *T. australis*, combined after extraction, was 54% of the sum of the activity of the two component enzymes when assayed independently. The analogous value of *Z. mays* and *P. dilatatum* was 61% and for *P. dilatatum* and *T. australis* 75%. Since the maize enzyme was *ca* 4-fold more active than the extract from the other species, this implies that at least the maize enzyme must have been subject to inhibition by the extracts from the other species. When *Z. mays* was co-extracted with *T. australis* and *Z. mays* co-extracted with *P. dilatatum* the activities were 50% and 68% of the sum of the activities when each species was extracted and assayed independently. Similarly the maize enzyme was shown to be inhibited by extracts from *Kalanchoe daigremontiana*, *Euphorbia pulcherrima*, *Capsicum annuum*, *Paspalum paspaloides*, *Pennisetum clandestinum*, *Sorghum bicolor*, *S. sudanense* and *Aloe arborescens* but not significantly (< 7%) by extracts from *Spinacia oleracea*, *Panicum milioides*, *Phalaris tuberosa*, *Egeria densa* and *Lagarosiphon major*.

Table 1. PPD activity of crude extracts of pre-illuminated leaf tissue

Family	Species	Classification	Reference	PPD activity (nmol/mg protein/min) With NH_4Cl	PPD activity (nmol/mg protein/min) Without NH_4Cl	Chlorophyll equivalent (μg/mg protein)
Gymnosperms						
Stangeriaceae	*Stangeria paradoxa*			6.7	4.2	37.2
Zamiaceae	*Bowenia spectabilis*			9.5	8.4	70.1
	Macrozamia communis			1.1	0	259
Dicotyledons						
Amaranthaceae	*Amaranthus caudatus*	C_4	[28]	23.5	7.8	173
	A. edulis	C_4	[28, 32]	26.9	8.6	171
	A. retroflexus	C_4	[28, 29]	56.9	47.2	210
	Gomphrena celosioides	C_4	[28, 33]	42.4	20.6	112
	G. globosa	C_4	[28, 29]	38.2	16.7	114
Crassulaceae	*Kalanchoe daigremontiana*	CAM	[19, 20]	1.2	0.3	46.2
Cruciferae	*Capsella bursa-pastoris*	C_3	[29]	0.3	0.1	79.7
	Raphanus sativus	C_3	[29]	0.2	0.1	156
Polygonaceae	*Rumex bidens*			0.2	0	930
Portulacaceae	*Portulaca oleracea*	C_4	[28, 29]	38.6	13.0	122
Solanaceae	*Capsicum annuum*			1.5	0.9	383
	Datura suaveolens			0.5	0	147
	Lycopersicon esculentum	C_3	[29]	0.6	0	136
	Solanum nigrum	C_3	[29]	0.4	0	295
	S. pseudocapsicum			0.2	0	184
Monocotyledons						
Gramineae	*Avena sativa*	C_3*	[28, 32]	3.6	2.1	120
	Bromus mollis			3.8	1.8	216
	Chloris gayana	C_4	[28, 29]	27.3	8.7	88.6
	Cynodon dactylon	C_4	[28, 29, 31]	35.8	6.8	145
	Digitaria sanguinalis	C_4	[28, 29]	19.6	3.8	230
	Ehrharta erecta	C_3	[31]	0.2	0.1	220
	Hordeum vulgare	C_3	[29, 31]	0.3	0.2	208
	Lolium perenne	C_3	[29]	2.9	1.9	42.7
	Panicum maximum	C_4	[28, 29]	58.9	17.9	146
	P. miliaceum	C_4	[28, 29]	44.3	12.6	50.4
	Paspalum dilatatum	C_4	[28, 29]	27.0	11.4	168
	P. paspaloides	C_4	[29]	23.0	9.5	220
	Pennisetum clandestinum	C_4	[28, 29]	19.5	11.4	115
	P. glaucum	C_4	[28, 29]	35.3	5.3	282
	Phleum pratense	C_3	[29]	0.7	0	323
	Poa annua	C_3	[29]	0.2	0.1	312
	Saccharum officinarum	C_4	[28, 29, 31, 34]	55.7	18.3	83
	Sorghum bicolor	C_4	[28, 29, 31]	16.4	4.2	265
	S. sudanense	C_4	[28, 29, 31]	19.4	8.4	247
	Stenotaphrum secundatum	C_4	[28, 31, 32]	29.1	12.4	155
	Themeda australis	C_4	[28, 31]	17.5	5.6	289
	Triticum aestivum	C_3*	[29, 31]	0.2	0.1	240
	Zea mays	C_4	[28, 29, 31, 34]	191.2	81.3	103
Hydrocharitaceae	*Egeria densa*			1.6	0	213
	Lagarosiphon major			1.0	0	191
Liliaceae	*Agapanthus africanus*			0.8	0.3	92.4
	Aloe arborescens	CAM	[19, 20]	0.6	0	40.1
Orchidaceae	*Cattleya* × Mary Jane			12.1	4.6	32.2
	Celogyne massangeana			0.4	0	46.5
	Cymbydium canaliculatum	CAM	[18]	80.5	25.1	46.5
	C. madidum	C_3	[18]	42.1	30.2	36.0
	C. suave	C_3	[18]	3.8	1.4	41.3
	Phaius tancarvillae			1.6	0.2	85.6
	Vanilla pamona			3.2	0.9	40.2
Xanthorrhoeaceae	*Lomandra longifolia*			4.1	3.5	99.7

* *Avena sativa* and *Triticum aestivum* also exhibit C_4 CO_2 assimilation, mainly in the developing grains and the first leaf [4].

Table 2. Species in which significant PPD activity was not detected* in extracts of illuminated leaf tissue

Family	Species	Classification	Reference
Filicinae			
Polypodiaceae	*Nephrolepis hirstula*		
Gymnosperms			
Pinaceae	*Pinus radiata*		
Dicotyledons			
Aizoaceae	*Mesembryanthemum crystallinum*	C_3/CAM	[19, 20]
Chenopodiaceae	*Beta vulgaris*	C_3	[34]
	Chenopodium album	C_3	[29, 33]
	Spinacia oleracea	C_3	[29]
Compositae	*Arctotheca calendula*		
	Cirsium vulgare	C_3	[29]
	Helianthus annuus	C_3	[29, 32]
	Lactuca sativa	C_3	[34]
	Sonchus oleraceus		
Cruciferae	*Brassica oleracea* cv *capitata*	C_3	[29]
	B. oleracea cv *italica*	C_3	[29]
	Raphanus raphanistrum		
Euphorbiaceae	*Euphorbia milli*	C_3	[35]
	E. peplus		
	E. pulcherrima	C_3	[29, 35]
Geraniaceae	*Pelargonium* × domesticum		
Leguminosae	*Kennedia rubicunda*		
	Medicago arabica		
	M. sativa	C_3	[29]
	Pisum sativum	C_3	[29, 32]
	Vicia faba	C_3	[29]
Magnoliaceae	*Magnolia grandiflora*	C_3	[32]
Myrtaceae	*Eucalyptus obliqua*		
	E. regnans		
Plantaginaceae	*Plantago lanceolata*	C_3	[29]
Polygonaceae	*Polygonum aviculare*	C_3	[29]
Primulaceae	*Anagallis arvensis*		
Solanaceae	*Nicotiana tabacum*	C_3	[29, 32]
	Solanum melongena	C_3	[29]
	S. tuberosum	C_3	[29]
Monocotyledons			
Gramineae	*Agrostis alba*	C_3	[29, 32]
	Avena fatua	C_3	[29]
	Briza minor		
	Dactylis glomerata	C_3	[29, 31]
	Festuca rubra	C_3	[29]
	Panicum milioides	†	
	Phalaris tuberosa	C_3	[31]
	Poa pratensis	C_3	[29, 31]
Liliaceae	*Allium triquetrum*		
	Doryanthes excelsa		
Orchidaceae	*Calanthe triplicata*	CAM	[18]

*PPi exchange values < 0.1 nmol/mg protein/min were not significant.

†The CO_2 assimilation mechanism of *Panicum milioides* is uncertain [26, 27, 29, 31]—see text.

PPD activity of epidermal strips of some C_3 species

The C_4 mechanism of CO_2 assimilation has been implicated in the process of stomatal opening in C_3 plants [13–15]. This would afford an explanation for the low level PPD activity associated with some C_3 plants, in which case most of the PPD associated with leaves would be localized in the epidermis. Extracts of whole leaves of *Agapanthus africanus* exhibited low level PPi incorporation which was enhanced by PEP and AMP and NH_4^+. Pretreatment of the tissue in the light enhanced the activity (Table 3). These features characterize the PPi incorporation activity as PPD. However, the rates and properties of the PPD activity associated with extracts

Table 3. PPD activity in extracts of whole leaves, epidermal strips and non-epidermal leaf tissue of *Agapanthus africanus* with and without illumination prior to extraction

Tissue	Incubation conditions	PPD activity* (nmol/mg protein/min)	
		Pre-illuminated	Dark
Whole leaves	Complete	0.67	0.40
	Without NH_4Cl	0.28	0.20
Epidermal strips	Complete	0.67	0.52
	Without NH_4Cl	0.51	0.50
Non-epidermal leaf tissue	Complete	0.62	0.24
	Without NH_4Cl	0.44	0.15

*PPD is shown corrected for PPi incorporation in incubations lacking AMP.

prepared from epidermal strips and non-epidermal material were similar to those of whole leaves, suggesting that the low level PPD activity found in this species does not have a specific location in epidermal tissue.

Properties of the low level PPD activity of oat leaf tissue

The PPi incorporation catalysed by crude extracts of oats (*Avena sativa*) exhibited characteristics similar to those described for maize [9]. They include the dependence on PEP and AMP, enhancement by NH_4^+ and an elevated level of extractable activity in tissue illuminated prior to extraction (Table 4). In addition the activity was irreversibly cold labile; activity decreased by 46% and 77% after storage for 24 hr and 48 hr at 22°, but by 72% and 90% after storage for 24 hr and 48 hr at 4° respectively. When crude extracts were subjected to $(NH_4)_2SO_4$ fractionation and ion exchange chromatography on DEAE-cellulose as used previously for the partial purification of maize leaf PPD [9], the oat enzyme was purified *ca* 16-fold. (Table 5). The final specific activity was *ca* 10% of the purified maize enzyme. The partially purified oat enzyme exhibited short term reversible cold lability (Fig. 2). Activity decreased by *ca* 50% in 10 min upon transferring the enzyme from 30° to 0°. However, unlike the PPD from maize cv Flat Red [9], the oat enzyme only recovered *ca* 75% of its initial activity when transferred to 30° after 130 min storage at 0°. This effect has also been observed for PPD from various maize cultivars [16].

Properties of the low level PPD activity of Capsicum annum

The PPi incorporation catalysed by crude extracts of *C. annum* was dependent on AMP and PEP, activity was enhanced by NH_4^+ and illumination of the tissue prior to extraction (Table 4). Crude extracts of *C. annum* strongly inhibited maize leaf PPD; the activity of the combined extracts was only 63% of the sum of the independent activities. The *C. annum* enzyme exhibited long term irreversible cold lability; the PPD activity of crude extracts stored for 24 hr at 22° and 4° decreased by 44% and 93% respectively. However the *C. annum* enzyme exhibited some features which distinguish it from the oat leaf enzyme. Upon fractionation of crude extracts with $(NH_4)_2SO_4$ most of the activity (72%) was associated with the 0–40% saturated fraction (cf. 77% in 40–50% saturated fraction for oats). In addition, the *C. annum* enzyme exhibited considerable short term irreversible cold lability.

Table 4. Substrate requirements and the effect of pre-illumination on the PPD activity of crude extracts of *Avena sativa* and *Capsicum annum*

Species	Assay conditions	PPi incorporated* (nmol/mg protein/min)	
		Pre-illuminated	Dark
A. sativa	Complete	7.52	2.61
	$-NH_4Cl$	5.56	2.36
	−AMP	0.68	0.52
	−PEP	1.06	0.90
C. annuum	Complete	0.61	0.34
	$-NH_4Cl$	0.41	0.23
	−AMP	0.05	0.03
	−PEP	0.04	0.03

*Values uncorrected for endogenous activity.

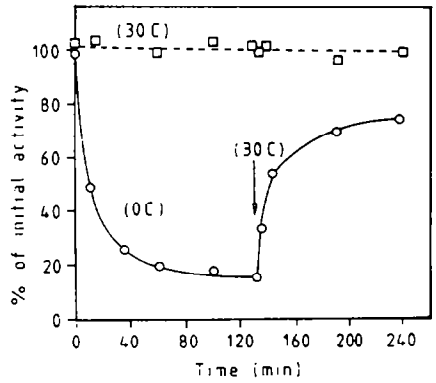

Fig. 2. Reversible cold lability of partially purified PPD from oats. The enzyme preparation was preincubated either at 30° (□) or 0° and subsequently at 30° (○) prior to determining PPD activity in standard assays at 30°.

DISCUSSION

The association of high PPi incorporation activity with plants of established C_4 status and the enhancement of this activity by NH_4^+ (Table 1) constitutes substantial evidence for attributing PEP plus PPi-dependent phosphorylation of AMP activity to PPD. However, although negligible PPD activity was detected in the leaves of many C_3 plants (Table 2), significant PPD activity was detected in many C_3 species, mostly grasses (Table 1). This confirms the recent results of Aoyagi and Bassham [7, 17] who documented the presence of PPD in the leaves of C_3 species using immunological procedures and partial purification of the enzyme. The presence of low level activity in some C_3 plants (Table 1) demonstrates that the PPD activity of leaf tissue must be used quantitatively rather than qualitatively for distinguishing C_3 and C_4 plants as also reported by Aoyagi and Bassham [7]. The possibility that the low level PPi incorporation activity in C_3 plants could be attributed to some process other than PPD is rendered unlikely by the requirement for both AMP and PEP, activation by NH_4^+, short term reversible cold inactivation and light activation, all of which are specific characteristics of PPD from C_4 plants. Furthermore, the oat enzyme was purified to the same extent as the enzyme from maize using the same procedures (Table 5 and ref. [9]).

Among the C_3 grasses, low but significant PPD activity was detected in *Avena sativa*, *Bromus mollis*, *Hordeum vulgare*, *Lolium perenne*, *Phleum pratense* and *Triticum aestivum*. The lack of any specific association of PPD with epidermal strips in *Agapanthus africanus* (Table 3) suggests that PPD is not specifically associated with the proposed C_4 metabolism of guard cells in this C_3 species. Furthermore, if all the PPD activity of the more active C_3 species such as *Avena sativa* was localized in the guard cells, this would result in an extremely high activity in these cells. It has been proposed that the low level PPD activity associated with the grains of C_3 grasses serves to generate PEP for use in the reassimilation (via PEP carboxylase) of CO_2 formed by respiration in the developing seed [6], and thereby contribute to the final grain yield, but the function of PPD in the leaves of C_3 plants is unknown.

Table 5. Purification of the activity catalysing PEP plus PPi-dependent phosphorylation of AMP from oat leaf tissue

Treatment	Protein (mg)	Specific activity (nmol/mg protein/min)
Crude extract	205	8
$(NH_4)_2SO_4$	41.3	30
DEAE-cellulose	8.4	122

In theory, the PPD activities associated with C_4, C_3 and CAM plants (Table 1) could be used to predict whether the C_4 mechanism of CO_2 assimilation is involved in species in which this is unknown since all C_4 species exhibit PPD activity > 15 nmol/mg protein/min (Table 1). However, the coincubation experiments of maize PPD with crude extracts from other species provides evidence for inhibitors of maize PPD in the extracts of these species. Presumably the PPD activity associated with the extracts of the other species is similarly affected, resulting in under estimation of PPD activity. The PPD activity of *Lomandra longifolia* is of interest in this regard. The leaf anatomy of this species exhibits characteristics similar to those of C_4 plants [Wong Hee, K. and Staff, I. A., personal communication] but the PPD activity in the tough, fibrous leaves was < 15 nmol/mg protein/min. Perhaps significantly, the PPD activity of this species was greater than all the known C_3 plants examined for PPD activity (Tables 1 and 2) although some extracts of *Avena sativa* showed activity up to 8 nmol/mg protein/min. Other factors which could contribute to the under-estimation of PPD activity in some species include the extraction and incubation conditions. For example the specific activity of the maize leaf enzyme is highly dependent on the enzyme concentration [9]. Here it is noteworthy that the conditions used to determine PPD activity were devised in a detailed study of the maize leaf enzyme [9]: the activity of extracts from this species was far in excess of those from all other species (Table 1). For some species the illumination conditions for short term light activation immediately prior to extraction and the irradiance during growth (Fig. 1) may not have been conducive for the maximum expression of PPD activity.

Uncertainty exists whether PPD activity can be used to establish the mechanism of CO_2 assimilation in orchids. *Cymbidium madidum*, *C. suave* and *Calanthe triplicata* exhibit δ-^{13}C discrimination values typical of C_3 plants but the value for *Cymbidium canaliculatum* is typical of a CAM plant [18]. However, of the former group only *C. suave* and *Calanthe triplicata* exhibit PPD activity < 15 nmol/mg/ protein/min (Table 1). *C. canaliculatum* showed very high PPD activity but other well-documented CAM species (eg. *Kalanchoe daigremontiana* and *Aloe arborescens*) contained much lower levels (Table 1). The CAM mechanism of CO_2 assimilation is common in orchids including *Cattleya* (13 species), *Vanilla* (two species) and *Dendrobium* (19 species) [18–20]. However, within the genus *Cymbidium* the products of CO_2 assimilation have only been examined in *Cymbidium* cv Cym-doris and found to be typical of the C_3

mechanism [21]. Since the high PEP plus AMP-dependent PPi incorporation activity of *C. madidum* and *C. canaliculatum* is not typical of well documented CAM plants and is also uncharacteristic of C_3 plants, this brings into question the function of PPD in these species. The PPD activity and the CO_2 assimilation patterns (and hence δ-^{13}C discrimination) in CAM plants are dependent on the specific growth conditions of the plants surveyed [22]. Collectively, these issues highlight the need for an extensive survey of the patterns of CO_2 incorporation in the Orchidaceae under a variety of growth regimes.

The PPD activity of several other species is also of interest. The mechanism of CO_2 assimilation in submerged aquatic macrophytes is a matter of some debate [23–26]. In the absence of any evidence for the inhibition of maize PPD by extracts of *Egeria densa* and *Lagarosiphon major*, the low level PPD activity in these species (Table 1) is consistent with the C_3 CO_2 assimilation mechanism. Similarly there has been considerable disagreement concerning the CO_2 assimilation mechanism in *Panicum milioides* [26, 27]. Extracts of *P. miliaceum* and *P. maximum*, both reportedly C_4 plants [28, 29], contained considerable PPD activity but significant activity was not detected in extracts of *P. milioides* suggesting that it possesses a C_3 pathway. The significant PPD activity in the gymnosperms *Bowenia spectabilis* and *Stangeria paradoxa* merit a detailed study of these and other gymnosperms for the existence of either the C_4 or CAM pathway.

EXPERIMENTAL

Whole plants or, where this was not possible, detached leaves were pre-illuminated for 2 hr before slicing and extracting under N_2 by method 2 described in ref. [9]. PPD activity was determined by PEP plus PPi-dependent phosphorylation of AMP using the assay conditions described for method B in ref. [9] except that activity was routinely determined in the presence of 50 mM NH_4Cl. Chlorophyll (Chl) was determined as in ref. [30] and all other procedures were as described previously [9].

Acknowledgements—C.G.H. was the holder of a Commonwealth Postgraduate Scholarship. The work reported in this article is derived from the resulting Ph.D. thesis (La Trobe University, 1981). We wish to thank Dr. M. D. Hatch, C.S.I.R.O. Division of Plant Industry, Canberra for generously supplying seeds of many of the C_4 species described in this paper.

REFERENCES

1. Edwards, G. and Walker, D. A. (1983) *C_3, C_4: Mechanisms, and Cellular and Environmental Regulation, of Photosynthesis*. Blackwell Scientific Publications, Oxford.
2. Hatch, M. D. and Slack, C. R. (1968) *Biochem. J.* **106**, 141.
3. Hatch, M. D. and Slack, C. R. (1975) *Methods Enzymol.* **42**, 212.
4. Wirth, E., Kelly, G. J., Fishbeck, G. and Latzko, E. (1977) *Z. Pflanzenphysiol.* **82**, 78.
5. Duffus, C. M. and Rosie, R. (1973) *Planta* **114**, 219.
6. Meyer, A. O., Kelly, G. J. and Latzko, E. (1978) *Plant Sci. Letters* **12**, 35.
7. Aoyagi, K. and Bassham, J. A. (1984) *Plant Physiol.* **75**, 387.
8. Sugiyama, T. (1973) *Biochemistry* **12**, 2862.
9. Hocking, C. G. and Anderson, J. W. (1985) *Phytochemistry* **24**, 2173.
10. Hatch, M. D. and Slack, C. R. (1969) *Biochem. J.* **112**, 549.
11. Yamamoto, E., Sugiyama, T. and Miyachi, S. (1974) *Plant Cell Physiol.* **15**, 987.
12. Hatch, M. D., Slack, C. R. and Bull, T. A. (1969) *Phytochemistry* **8**, 697.
13. Willmer, C. M. and Dittrich, P. (1974) *Planta* **117**, 123.
14. Rashke, K. and Dittrich, P. (1977) *Planta* **134**, 69.
15. Schnabl, H. (1981) *Planta* **152**, 307.
16. Sugiyama, T. and Boku, K. (1976) *Plant Cell Physiol.* **17**, 851.
17. Aoyagi, K. and Bassham, J. A. (1983) *Plant Physiol.* **73**, 853.
18. Winter, K., Wallace, B. J., Stocker, G. C. and Roksandie, Z. (1983) *Oecologia* **57**, 129.
19. Black, C. C. and Williams, S. (1976) in CO_2 *Metabolism and Plant Productivity* (Burris, R. H. and Black, C. C., eds) p. 407. University Park Press, Baltimore.
20. Szarek, S. R. and Ting, I. P. (1977) *Photosynthetica* **11**, 330.
21. Hatch, M. D., Slack, C. R. and Johnson, H. S. (1967) *Biochem. J.* **102**, 417.
22. Holtum, J. A. M. and Winter, K. (1982) *Planta* **155**, 8.
23. Stanley, R. A. and Naylor A. W. (1972) *Plant Physiol.* **50**, 149.
24. De Groote, D. and Kennedy, R. A. (1977) *Plant Physiol.* **59**, 1133.
25. Browse, J. A., Fromgoole, F. I. and Brown, J. M. (1977) *Aust. J. Plant Physiol.* **4**, 169.
26. Hattersley, P. W., Watson, L. and Osmond, C. B. (1977) *Aust. J. Plant Physiol.* **4**, 523.
27. Kanai, R. and Kashiwagi, M. (1975) *Plant Cell Physiol.* **16**, 669.
28. Downton, W. J. (1975) *Photosynthetica* **9**, 96.
29. Krenzer, E. G., Moss, D. N. and Crookston, R. K. (1975) *Plant Physiol.* **56**, 194.
30. Jeffrey, S. W. and Humphrey, G. F. (1975) *Biochem. Physiol. Pflanz.* **167**, 191.
31. Smith, B. N. and Brown, W. V. (1973) *Am. J. Botany* **60**, 505.
32. Smith, B. N. and Epstein, S. (1971) *Plant Physiol.* **47**, 380.
33. Johnson, H. S. and Hatch, M. D. (1968) *Phytochemistry* **7**, 375.
34. Hatch, M. D., Slack, C. R. and Johnson, H. S. (1967) *Biochem. J.* **102**, 417.
35. Webster, G. L., Brown, W. V. and Smith, B. N. (1975) *Taxon* **24**, 27.