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DISTRIBUTION OF THE C_4-DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS AND ITS OCCURRENCE IN DICOTYLEDONOUS PLANTS

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Abstract—The C_4-dicarboxylic acid pathway of photosynthesis was originally shown to operate in certain monocotyledons including many tropical grasses, but not in several dicotyledons. The present paper reports the operation of this pathway in genera from two dicotyledonous families, Amaranthaceae (*Amaranthus* and *Gomphrena*) and Chenopodiaceae (*Atriplex*). Further investigation of the monocotyledonous family Cyperaceae has revealed that the pathway also occurs in several species of *Cyperus*, and a species of *Kyllinga*, but not in *Cyperus gracilis*. In these plants the occurrence of the C_4-dicarboxylic acid pathway of photosynthesis correlates with low ribulose diphosphate carboxylase activity, high activities of phosphopyruvate carboxylase and phosphopyruvate synthetase, a unique type of leaf anatomy, and high photosynthesis rates.

INTRODUCTION

THE $^{14}CO_2$ fixed under steady-state conditions of photosynthesis in leaves of tropical grasses appears initially in the C-4 of oxaloacetate, malate and aspartate.[1,2] From time course studies on $^{14}CO_2$ fixation[1] and subsequent comparative studies on enzyme activities[3] it was concluded; (1) that the primary carboxylation reaction is catalyzed by phosphopyruvate carboxylase; (2) that the C-4 carboxyl of oxaloacetate is transferred to an acceptor, yielding carboxyl-labelled 3-phosphoglycerate; and (3) that the pyruvate derived from the C-1, C-2, and C-3 of oxaloacetate serves to regenerate phosphopyruvate, the initial CO_2 acceptor. In support of the latter conclusion, a new enzyme which catalyzes the conversion of pyruvate to phosphopyruvate has been found in extracts of tropical grass leaves but not in leaves in which the Calvin Cycle is operative.[4,5]

A previous survey[2] established that the photosynthetic pathway which features dicarboxylic acids as early products was operative in some monocotyledonous plants including several tropical grasses and a species of *Cyperus*, but not in other monocotyledons or in several dicotyledonous plants. The present paper provides evidence for the operation of the C_4-dicarboxylic acid pathway of photosynthesis in species of the dicotyledons, *Amaranthus* and *Gomphrena* (Amaranthaceae) and *Atriplex* (Chenopodiaceae) and in several other species of the monocotyledons, *Cyperus* and *Kyllinga* (Cyperaceae).

[1] M. D. HATCH and C. R. SLACK, *Biochem. J.* **101**, 103 (1966).
[2] M. D. HATCH, C. R. SLACK and HILARY S. JOHNSON, *Biochem. J.* **102**, 417 (1967).
[3] C. R. SLACK and M. D. HATCH, *Biochem. J.* **103**, 660 (1967).
[4] M. D. HATCH and C. R. SLACK, *Arch. Biochem. Biophys.* **120**, 224 (1967).
[5] M. D. HATCH and C. R. SLACK, *Biochem. J.*, in press (1968).

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN INDIVIDUAL COMPOUNDS AFTER PHOTOSYNTHESIS IN $^{14}CO_2$ FOR APPROXIMATELY FOUR SECONDS

Species	% of total radioactivity in individual compounds					
	Oxaloacetate*	Malate	Aspartate	3-Phospho-glycerate	Sugar phosphates	Other compounds
Amaranthaceae						
Amaranthus viridis	0·14	22	64	6	0	8
Amaranthus palmeri	—	20	69	11	0	0
Gomphrena celosoides	2	45	38	8	4	3
Chenopodiaceae						
Atriplex semibaccata	0·05	37	52	5	0	5
Chenopodium album	0	0	0	90	10	0
Cyperaceae						
Cyperus species†	—	57–75	25–34	0–10	0–5	0–3
Kyllinga monocephala	—	71	15	10	0	4
Other genera of the Cyperaceae‡	0	0	0	38–43	30–62	0–20

* Oxaloacetate was determined as its 2,4-dinitrophenylhydrazone as described in the Methods section.

† *Cyperus rotundus*, *C. polystachyos* and *C. bowmannii*. The results shown are the extreme values obtained for individual plants.

‡ *Gahnia sp.*, *Lepidosperma sp.* and *Cyperus gracilis*.

RESULTS AND DISCUSSION

Incorporation of $^{14}CO_2$ into Leaves

When species of *Atriplex*, *Amaranthus*, *Gomphrena*, *Kyllinga*, and *Cyperus* leaves had attained a steady rate of photosynthesis at light-saturation in air, exposure to $^{14}CO_2$ for 4 sec resulted in most of the fixed radioactivity being located in C_4-dicarboxylic acids (Table 1). These acids, malate, aspartate and oxaloacetate, are assumed to be rapidly interconverted.[1] Only a small proportion of radioactivity was located in 3-phosphoglycerate and hexose phosphates. This pattern of labelling closely resembles that obtained previously for tropical grasses treated in a similar manner but contrasts with the pattern obtained for several other monocotyledons and dicotyledons.[2] With the latter plants, and with the plants examined during the present studies including *Cyperus gracilis* and species of two other genera of the

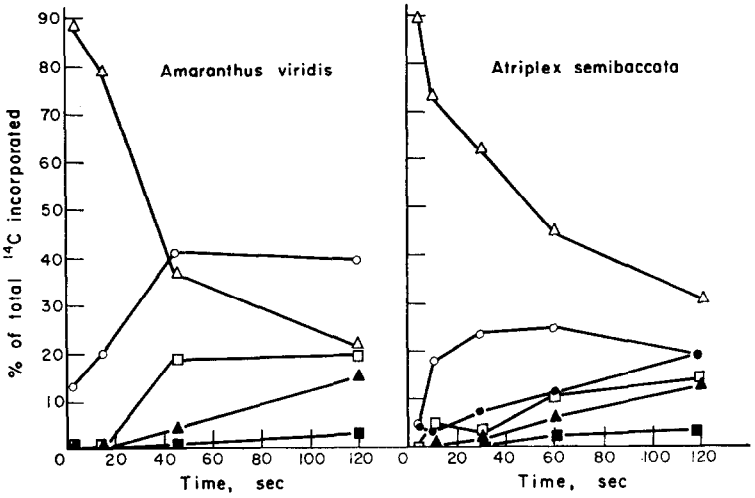

FIG. 1. PROPORTION OF THE TOTAL RADIOACTIVITY IN INDIVIDUAL COMPOUNDS AFTER DIFFERENT PERIODS IN $^{14}CO_2$.

The compounds are malate plus aspartate (△), 3-phosphoglycerate (○), sugar phosphates (□), sucrose (▲), α-glucan (■) and alanine (●).

family Cyperaceae, and with *Chenopodium album*, the labelling patterns were typical of the operation of the Calvin Cycle. From the studies to date variation in the photosynthetic pathway within a genus has only been observed in *Cyperus*.

Time-course studies on $^{14}CO_2$ incorporation into leaves of the dicotyledons *Atriplex semibaccata* and *Amaranthus viridis* provided information about the steps subsequent to the labelling of the dicarboxylic acids. With increasing time the proportion of radioactivity in the C_4-dicarboxylic acids declined with concomitant increases in 3-phosphoglycerate, sugar phosphates, sucrose and polysaccharide (Fig. 1). A labelling sequence of this type was originally shown in sugar cane leaves[1,6] and the movement of radiocarbon from the dicarboxylic acids to sugars was directly demonstrated by transferring leaves from $^{14}CO_2$ to $^{12}CO_2$.[1] Similarly interpreted, the results for *Atriplex* and *Amaranthus* are consistent with radiocarbon from $^{14}CO_2$ being incorporated into the C_4-dicarboxylic acids from which it is

[6] H. P. KORTSCHAK, C. E. HARTT and G. O. BURR, *Plant Physiol.* **40**, 209 (1965).

subsequently transferred via 3-phosphoglycerate to hexose phosphates, sucrose and polysaccharide. Osmond[7] observed similar early products with *Atriplex spongiosa* but found no decline in the percentage of the fixed radioactivity in the C_4-dicarboxylic acids with concomitant rises in 3-phosphoglycerate and hexose phosphates with time. However in the latter studies the time-course data were accumulated from several separate experiments conducted under non-steady state conditions.

With *Amaranthus* and *Atriplex* nearly 100 per cent of the radioactivity in aspartate after 4 sec in $^{14}CO_2$ was located in the C-4. After 120 sec the percentages in the C-4 were 50 and 35 per cent in *Atriplex* and *Amaranthus* respectively. These results are consistent with those obtained for sugar cane[1] in which it was proposed that only radioactivity from the C-4 of dicarboxylic acids appears in 3-phosphoglycerate. From studies with sugar cane[1,3] it was concluded that the slow labelling of the C-1, C-2 and C-3 of aspartate proceeded by an exchange of radioactivity between radioactive 3-phosphoglycerate and phosphopyruvate, the latter being the direct precursor of these carbons.

Enzyme Activities and Photosynthesis Rates

Several tropical grasses and *Amaranthus palmeri* have photosynthetic rates at least twice that of the temperate grasses and other monocotyledons and dicotyledons.[3,8] During the present studies similar rates were obtained for *Amaranthus palmeri* and *Atriplex semibaccata* (Table 2). In *Amaranthus* and *Atriplex* leaf extracts, the activity of ribulose diphosphate

TABLE 2. ENZYME ACTIVITIES AND PHOTOSYNTHESIS RATES IN *Amaranthus* AND *Atriplex* SPECIES COMPARED WITH OTHER PLANTS

Tissue	Activity (μmoles/mg chlorophyll/min)			
	Maximum photosynthesis rate*	Ribulose 1,5 diphosphate carboxylase	Phosphopyruvate carboxylase	Phosphopyruvate synthetase
Amaranthus palmeri	4·8	0·6	21	3·7
Atriplex semibaccata	4·0†	0·2	18	1·4
Tropical grasses‡	2·9–3·5	0·3–0·6	15–18	1·2–3·5
Calvin Cycle plants‡	1·6–1·7	4·2–4·7	0·3–0·35	0

* Measured at light-saturation and 0·03 per cent (v/v) carbon dioxide.

† Measured by M. M. Ludlow, Department of Botany, University of Queensland.

‡ The results for the tropical grasses (sugar cane, maize and sorghum) and Calvin Cycle plants (wheat, oat and silverbeet) are those reported previously[3,5] and are the extremes of values for individual plants.

carboxylase was much lower than that observed in Calvin Cycle plants and was of the order of $\frac{1}{10}$ of the photosynthetic rates recorded for these leaves (Table 2). Its activity was of the same order as that observed for the tropical grasses.[3] In contrast, the activity of phosphopyruvate carboxylase in these leaves and in tropical grasses was in excess of their maximum photosynthetic rates. The enzyme which catalyzes the conversion of pyruvate to phosphopyruvate, referred to as phosphopyruvate synthetase,[5] was also present in extracts of *Amaranthus* and *Atriplex* leaves. Because of the unusual instability of this enzyme[5] it would be reasonable to regard the recorded activities as minimal. *Cyperus* leaves apparently contain

[7] C. B. OSMOND, *Biochem. Biophys. Acta* **141**, 197 (1967).

[8] M. EL-SHARKAWY and J. HESKETH, *Crop Sci.* **5**, 517 (1965).

a protein-precipitating factor since aqueous extracts prepared by our standard procedure contained virtually no protein and no detectable enzyme activities. However with extracts of acetone powders of *Cyperus rotundus* leaves activities of 2·2 and 0·9 μmoles/mg chlorophyll/min were obtained for phosphopyruvate carboxylase and phosphopyruvate synthetase respectively.

Leaf Anatomy

Prat[9] and other workers[10] have divided the grasses into two main groups, Panicoid (tropical) and Festucoid (temperate), on the basis of their leaf anatomy. In the former group, the chlorenchyma cells are radially arranged around the vascular bundles, the majority of chloroplasts appearing in the bundle sheath parenchyma. In the latter group they are irregularly arranged between adjacent vascular bundles. All the grasses in which the C_4-dicarboxylic acid pathway of photosynthesis has been demonstrated belong to the Panicoid group.[2] This distinctive radial arrangement of chloroplast containing cells, was demonstrated in *Amaranthus palmeri*[11] and by us in *Atriplex semibaccata, Gomphrena celosoides, Kyllinga monocephala* and *Cyperus rotundus*. Of the several plants examined in which the Calvin Cycle is operative, including *Cyperus gracilis*, none had this type of leaf anatomy.

Concluding Comments

Hitherto, the photosynthetic process which results in the rapid labelling of the C_4-dicarboxylic acids has been demonstrated in Panicoid grasses[1,2] and one species of *Cyperus* but no dicotyledonous plants. The occurrence of this pathway is associated with several unique differences in enzyme activities.[3,5] With the demonstration of these characteristics in species of two dicotyledonous families and in several other species of the family Cyperaceae, it now appears that the C_4-dicarboxylic acid pathway of photosynthesis will have a relatively wide distribution within the higher plant kingdom.

The two groups of plants, separable by their differing early photosynthetic products and photosynthetic enzymes also appear to differ in several other respects. In addition to the variation in leaf anatomy already referred to, there are recent reports of differences in chloroplast structure,[12] maximum photosynthesis rates and light-saturation values,[8] photorespiration and compensation point,[13] and the effects of oxygen and inhibitors on photosynthesis rates.[14,15] The significance of the relationships between the operation of the C_4-dicarboxylic acid pathway of photosynthesis and these other features remain to be resolved.

MATERIALS AND METHODS

Studies were conducted with mature leaves of plants collected from the field or grown in a greenhouse. The sources of radiochemicals, biochemicals and reagent enzymes were as previously described.[3,5]

The procedures for studying the incorporation of $^{14}CO_2$ into leaves and for the identification of the radioactive products were as described previously.[1,2] Oxaloacetate was isolated

[9] H. PRAT, *Ann. Sci. Nat. Botan.* Ser. **10**, 18, 165 (1936).
[10] W. V. BROWN, *Botan. Gaz.* **119**, 170 (1958).
[11] J. HESKETH, personal communication.
[12] SISTER M. C. JOHNSON, Ph.D. Thesis, University of Texas, Austin, Texas, U.S.A. (1964).
[13] M. L. FORRESTER, G. KROTKOV and C. D. NELSON, *Plant Physiol.* **41**, 422 (1966).
[14] M. L. FORRESTER, G. KROTKOV and C. D. NELSON, *Plant Physiol.* **41**, 428 (1966).
[15] I. ZELITCH, *Plant Physiol.* **41**, 1623 (1966).

as its 2,4-dinitrophenylhydrazone after killing the leaves in an ethanolic solution at −80° containing 2,4-dinitrophenylhydrazine.[2] Aspartate was purified by eluting the aspartate area from paper chromatograms developed with butan-1-ol-propionic acid-water (10:5:3)[16] and re-chromatographing with the solvent, isoamyl alcohol-pyridine-water:diethylamine (10:10:7:0·3)[17] to remove contaminating sucrose and amino acids. The distribution of radioactivity in aspartate was determined after conversion to oxaloacetate with aspartate aminotransferase and an excess of 2-oxoglutarate. Treatment of the oxaloacetate formed with $CuSO_4^3$ gave the C-4 carboxyl as $^{14}CO_2$ and the C-1, C-2, and C-3 carbons as pyruvate.

Alanine was identified from extracts of *Atriplex semibaccata* by elution of the compound from paper chromatograms developed with butan-1-ol-propionic acid-H_2O (10:5:3) followed by co-chromatography of this sample with unlabelled alanine in phenol-H_2O (1 kg phenol in 395 ml H_2O)[18] and pyridine-H_2O (80:20).[19] The compound was degraded to volatile radioactive components following treatment with a ninhydrin reagent.[2]

Enzyme activities were determined with leaf extracts prepared and treated on a small column of Sephadex G-25 as previously described,[3] except that dithiothreitol was used instead of 2-mercaptoethanol. Procedures for the assay of phosphopyruvate carboxylase and ribulose diphosphate carboxylase[3] and for phosphopyruvate synthetase[5] were those used previously. Chlorophyll was determined by the procedure of Arnon.[20]

[16] A. A. BENSON, J. A. BASSHAM, M. CALVIN, T. C. GOODALE, V. A. HAAS and W. STEPKA, *J. Am. Chem. Soc.* **72**, 1710 (1950).
[17] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *Paper Chromatography and Paper Electrophoresis*, p. 154, 2nd edition Academic Press, New York (1958).
[18] W. STEPKA, In *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 3, p. 517. Academic Press, New York (1957).
[19] J. A. BASSHAM and M. KIRK, *Biochem. Biophys. Acta* **90**, 553 (1964).
[20] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).