Phytochemistry, 1968, Vol. 7, pp. 581 to 584. Pergamon Press. Printed in England

DISTRIBUTION OF CARBONIC ANHYDRASE IN RELATION TO THE C_4 PATHWAY OF PHOTOSYNTHESIS

R. G. EVERSON and C. R. SLACK

The Colonial Sugar Refining Co. Ltd., David North Plant Research Centre, Indooroopilly, Brisbane, Queensland, Australia

(*Received* 19 *October* 1967)

Abstract—The level of carbonic anhydrase in leaves of species which fix CO_2 via the C_4-dicarboxylic acid pathway of photosynthesis is one-fifth to one-tenth that in species in which the normal Calvin cycle is operative. Analysis of non-aqueous fractions of leaves indicates that carbonic anhydrase is a cytoplasmic enzyme in plants with C_4-dicarboxylic acid pathway, whereas in plants with only the Calvin cycle the enzyme is located in the chloroplasts.

INTRODUCTION

ALTHOUGH the role of carbonic anhydrase in animals is well documented its physiological function in plants is still obscure. Carbonic anhydrase is present only in leaves[1,2] and has been implicated in the process of photosynthetic CO_2 fixation,[3] yet it is difficult to visualize its precise role in view of the report that the enzyme is not associated with chloroplasts.[2]

Our interest in carbonic anhydrase was stimulated by the recent report[4] that the enzyme was absent from leaves of maize, sorghum and sudan grass. These species together with other panicoid grasses differ from other plant species in certain as yet unrelated aspects of photosynthesis. Panicoid grasses exhibit higher maximum rates of CO_2 fixation,[5] have a CO_2 compensation point of zero,[6,7] have a different complement of certain photosynthetic enzymes,[8] and fix CO_2 into sugars via the C-1 of a C_4 dicarboxylic acid.[9,10] In the present paper we report that leaves of panicoid grasses do contain carbonic anhydrase but in considerably smaller amounts than other species, and present evidence that the carbonic anhydrase of panicoid grasses is not a chloroplast enzyme whereas in other species the enzyme is associated with chloroplasts.

RESULTS

Activity of Carbonic Anhydrase in Leaves

Leaf carbonic anhydrase is a sulphydryl enzyme requiring the presence of a sulphydryl-protecting agent for its successful isolation.[1] Hence, in the present studies leaves were

[1] J. R. G. BRADFIELD, *Nature* **159**, 497 (1947).
[2] E. R. WAYGOOD and K. A. CLENDENNING, *Can. J. Res.* **C28**, 673 (1950).
[3] G. O. BURR, *Proc. Roy. Soc. (London) Ser.* **B 120**, 42 (1936).
[4] J. D. HESKETH, H. MURAMOTO and M. A. EL-SHARKAWY, Report No. 2 on Photosynthesis, Dept. of Plant Breeding, Univ. of Arizona, Tucson (1965).
[5] J. D. HESKETH and D. N. MOSS, *Crop Sci.* **3**, 107 (1963).
[6] M. L. FORRESTER, G. KROTKOV and C. D. NELSON, *Plant Physiol.* **41**, 428 (1966).
[7] J. D. HESKETH, personal communication.
[8] C. R. SLACK and M. D. HATCH, *Biochem. J.* **103**, 660 (1967).
[9] M. D. HATCH and C. R. SLACK, *Biochem. J.* **101**, 103 (1966).
[10] M. D. HATCH, C. R. SLACK and H. S. JOHNSON, *Biochem. J.* **102**, 417 (1967).

homogenized under N_2 with 2-mercaptoethanol. The presence of Polyclar AT which prevents the inactivation of certain enzymes by phenolic compounds[11] did not increase the activity of carbonic anhydrase in the species investigated.

Contrary to the findings of Hesketh *et al.*[4] we detected carbonic anhydrase activity in leaf extracts of all panicoid grasses examined. However, in these species the activity of the enzyme was considerably less than in all other species examined except the members of the Amaranthaceae (Table 1). It is noteworthy that these species of *Amaranthus* and *Gomphrena* are similar to the panicoid grasses in other respects, having a high maximum rate of CO_2 fixation, zero CO_2 compensation point[12, 13] and a similar pathway of photosynthetic CO_2 fixation.[14]

TABLE 1. ACTIVITY OF CARBONIC ANHYDRASE IN LEAF EXTRACTS OF VARIOUS SPECIES

Family (subfamily)	Plant	Carbonic anhydrase units
Chenopodiaceae	*Spinacea oleracea*	3800
	Beta vulgaris	2200
Compositae	*Lactuca sativa*	1700
Leguminosae	*Pisum sativum*	4625
Amaranthaceae	*Amaranthus palmeri*	387
	Gomphrena globosa	520
Gramineae (Festucoideae)	*Triticum sativum*	1880
	Avena sativa	2200
(Panicoideae)	*Saccharum officinarum*	380
	Zea mays	350
	Sorghum hybrid	260

Leaf extracts were prepared and enzyme assayed as described in experimental section. Enzyme units expressed as $10\frac{(tb/te-1)}{\text{mg chlorophyll}}$ where *tb* and *te* are the times required for the indicator to change from blue to yellow in the absence and presence of enzyme respectively.

Intracellular Distribution of Carbonic Anhydrase in Leaves

Waygood and Clendenning[2] reported that leaf carbonic anhydrase is not associated with chloroplasts. However, the aqueous extraction procedure they employed to study the enzyme's location is open to question in view of later work[15, 16] showing that aqueously isolated chloroplasts lose a large proportion of certain enzymes. In the present studies we used a non-aqueous fractionation technique which has been successfully employed to study the intracellular location of leaf enzymes.[15, 16]

It is assumed that enzymes originally associated with chloroplasts, but not other enzymes, will remain associated with the chloroplasts during drying and fractionation and hence exhibit a distribution amongst the various fractions similar to that of chlorophyll.

[11] W. D. LOOMIS and J. BATTAILE, *Phytochem.* 5, 423 (1966).
[12] M. A. EL-SHARKAWY, R. S. LOOMIS and W. A. WILLIAMS, *Physiol. Plantarum* 20, 171 (1967).
[13] E. B. TREGUNNA and J. DOWNTON, *Can. J. Botany*, in press.
[14] H. S. JOHNSON and M. D. HATCH, *Phytochem.*, in press.
[15] C. R. STOCKING, *Plant Physiol.* 34, 56 (1959).
[16] R. M. SMILLIE and R. C. FULLER, *Plant Physiol.* 34, 651 (1959).

The results obtained with leaves of two species, pea and spinach, which contain high levels of carbonic anhydrase, and two, maize and *Amaranthus*, which contain low levels of the enzyme are shown in Table 2. The distribution of carbonic anhydrase is compared with that of chlorophyll, a chloroplast enzyme (ribulose diphosphate carboxylase), and a cytoplasmic enzyme (acid phosphatase). The carbonic anhydrase of both pea and spinach leaf fractionated in a manner characteristic of a chloroplast enzyme since its distribution between the various fractions was very similar to that of chlorophyll and ribulose diphosphate carboxylase, but different from that of acid phosphatase. In contrast, the small amount of carbonic anhydrase in maize and *Amaranthus* leaf behaved as a cytoplasmic enzyme fractionating differently from chlorophyll and ribulose diphosphate carboxylase.

The present data do not enable us to distinguish whether pea and spinach leaves contain a small fraction of their carbonic anhydrase associated with the cytoplasmic fraction.

TABLE 2. DISTRIBUTION OF ENZYMES AND CHLOROPHYLL IN LEAF FRACTIONS PREPARED IN NON-AQUEOUS MEDIA

Species	Fraction density	Chlorophyll % total	Enzyme activity (% total activity)		
			Carbonic Anhydrase	Ribulose diphosphate carboxylase	Acid phosphatase
Spinacea oleracea	<1·34	68	67	73	30
	1·34–1·40	20	22	15	14
	>1·40	12	11	12	56
Pisum sativum	<1·34	78	74	80	14
	1·34–14·0	16	18	14	13
	>1·40	6	8	6	73
Zea mays	<1·37	76	44	72	38
	1·37–1·40	10	16	14	20
	>1·40	14	41	14	42
Amaranthus palmeri	<1·35	87	36	85	18
	1·35–1·40	3	6	5	3
	>1·40	9	58	10	79

Leaf dried, fractionated and enzymes assayed as described in the Experimental section.

DISCUSSION

The above results indicate that in both the amount and the intracellular location of carbonic anhydrase the panicoid grasses and the members of the Amaranthaceae differ from the other species examined. The level of the enzyme was considerably higher in species which carry out photosynthesis via the normal Calvin cycle than in those in which the C_4-dicarboxylic acid pathway of photosynthesis operates.[10]

The presence of carbonic anhydrase in chloroplasts of species of the former group suggests that this enzyme may be required for the successful operation of the Calvin cycle. Enns[17] has recently demonstrated, using a model system, that carbonic anhydrase will enhance the rate of transport of CO_2 across a membrane by as much as 100-fold. In plants therefore carbonic anhydrase may act as a carrier of CO_2 maintaining the internal bicarbonate pool of chloroplasts during photosynthesis. Alternatively carbonic anhydrase may function in close

[17] T. ENNS, *Science* **155**, 44 (1967).

association with ribulose diphosphate carboxylase if CO_2 rather than HCO_3^- is the C_1 substrate for the carboxylase reaction.[18]

The absence of carbonic anhydrase from the chloroplasts of species in which the C_4-dicarboxylic acid pathway operates suggests that the fixation of CO_2 into a C_4 acid may serve a function in the leaf similar to that of carbonic anhydrase. It is noteworthy that phosphopyruvate carboxylase which is mainly responsible for the fixation of CO_2 into C_4 acids has a K_m for HCO_3^- of 0·4 mM[19] and hence would be expected to be a more efficient scavenger for HCO_3^- than ribulose diphosphate-carboxylase.

The zero CO_2 compensation point of maize leaves has been attributed to the absence of photorespiration[20] and that of *Amaranthus* to a highly efficient system for refixing HCO_3^- produced in photorespiration.[21] We suggest that both the low levels of carbonic anhydrase in these species and the high affinity of phosphopyruvate carboxylase for HCO_3^- would act against the loss of HCO_3^- produced in photorespiration.

EXPERIMENTAL

Plant Material

Young expanding leaves were obtained from plants grown in vermiculite culture in a glasshouse.

Preparation of Leaf Extracts

Leaf lamina (4g) was homogenized under N_2 in 20 ml of 0·1 M tris-HCl buffer (pH 8·3) containing 0·001 M EDTA and 0·01 M 2-mercaptoethanol. The homogenate was filtered through wetted miracloth (Chicopee Mills, Inc. N.Y.), after removing sufficient of the filtrate for chlorophyll determination[22] the remainder was centrifuged at 18,000 × *g* for 5 min. A sample of the supernatant (1 ml) was passed through a Sephadex-G25 column (12 × 0·7 cm) equilibrated with 0·0025 M Veronal buffer (pH 8·2) containing 0·005 M 2-mercaptoethanol and the protein collected in 1·2 ml. Eluates were assayed for carbonic anhydrase activity within 30 min of homogenizing the leaf. All operations were carried out at 4°.

Fractionation of Leaf by the Non-Aqueous Procedure

Leaves were destarched by holding plants at low light intensity for 24 hr, then frozen and freeze-dried as described by Stocking[15] and the dried leaf homogenized in a hexane-carbon tetrachloride mixture and fractionated by density as described by Smillie.[23]

Carbonic Anhydrase Assays

Carbonic anhydrase activity in leaf extracts prepared by the aqueous method was assayed by the Veronal-indicator method as described by Rickli *et al.*[24] When assaying the enzyme in fractions prepared by the non-aqueous method the volume of the reaction mixture was reduced and the procedure modified. Each fraction was suspended in a standard aliquot of 0·0025 M Veronal buffer (pH 8·2) containing 0·005 M 2-mercaptoethanol and assayed immediately. Reactions were carried out at 4° in a micro test tube (25 × 5 mm) containing a small glass bead and started by adding 0·10 ml CO_2-saturated water to 0·1 ml of the enzyme suspension and 0·15 ml of 0·025 M Veronal buffer (pH 8·2) containing bromothymol blue 2 mg/100 ml. The tube was capped and the reaction stirred by allowing the glass bead to roll through the liquid. The time taken for the indicator to change from blue to yellow was noted in the presence of boiled (*tb*) and unboiled (*te*) enzyme suspension. *Tb* was approximately 100 sec and *te* was adjusted to about 20 sec by appropriate dilution of the enzyme. Units of activity in each fraction were calculated from the formula $U = 10(tb/te - 1)$ and with other enzymes assayed expressed as percentage of total activity in a leaf sample.

Ribulose diphosphate-Carboxylase and Acid Phosphatase Assays

The enzymes were assayed as previously described.[8]

[18] A. WEISSBACH, B. L. HORECKER and J. HURWITZ, *J. Biol. Chem.* **218**, 795 (1956).
[19] Unpublished data.
[20] E. B. TREGUNNA, *Science* **151**, 1239 (1966).
[21] M. A. EL-SHARKAWY, R. S. LOOMIS and W. A. WILLIAMS, *Physiol. Plantarum* **20**, 171 (1967).
[22] D. I. ARNON, *Plant Physiol.* **24**, 1 (1949).
[23] R. M. SMILLIE, *Can. J. Botany*, **41**, 123 (1963).
[24] E. E. RICKLI, S. A. S. GHAZANFAR, B. H. GIBBONS and J. T. EDSALL, *J. Biol. Chem.* **239**, 1065 (1964).