

# Biochemistry of Photosynthesis in Species of *Triticum* of Differing Ploidy

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## ABSTRACT

Illuminated flag leaves of *Triticum monococcum*(2X), *T. urartu*(2X), *T. dicoccum*(4X), *T. dicoccoides*(4X), and *T. aestivum*(6X) were exposed to <sup>14</sup>CO<sub>2</sub> for 10 seconds and subsequently allowed to continue photosynthesis in the ambient air for periods of up to 2 minutes. The relative distribution of <sup>14</sup>C among water-soluble products in the leaves was similar for each species at each sampling time. After the 10-second pulse of <sup>14</sup>CO<sub>2</sub>, radioactivity was mainly in phosphate esters with less than 5% in C<sub>4</sub> acids. Subsequently, radioactivity increased in sucrose, glycine, and serine at the expense of that in phosphate esters. By 2 minutes, between 18% and 29% of the <sup>14</sup>C was in glycine plus serine. The results suggest rapid photorespiration in all species and an absence of C<sub>4</sub> photosynthesis.

D-Ribulose 1,5-bisphosphate carboxylase (EC 4.1.1.39) was partly purified from seedling leaves of each of the five *Triticum* species. Each preparation was assayed for simultaneous carboxylase and oxygenase activities in 2.1 millimolar NaHCO<sub>3</sub> and 265 micromolar O<sub>2</sub> at pH 8.2 and 25°C. The mean ratio of carboxylase to oxygenase activities was 6.11 ± 0.16 (standard error); differences between values for different species were not statistically significant. The results do not explain the faster rates of photosynthesis per unit leaf area reported for diploid and tetraploid species of *Triticum* compared to the hexaploid.

Modern cultivars of the hexaploid crop species *Triticum aestivum* produce larger yields per hectare of grain than the more primitive diploid and tetraploid species, but have slower rather than faster rates of net photosynthesis per unit leaf area (11). Thus far harvest index, rather than photosynthetic rate per unit leaf area, has been the major factor in the selection of wheats for increased yield (9, 17). It is logical however, that once the harvest index has been optimized, further improvements will require increased rates of photosynthesis by the leaves (1).

High rates of photosynthesis per unit leaf area have been correlated with several anatomical features including small leaf size, small mesophyll cell size, and close spacing of veins (8, 28). Recent evidence from comparative studies of the anatomy and morphology of the leaves of diploid, tetraploid, and hexaploid wheats demonstrated the special importance of cell size (15). But biochemical events comprise the major part of the total resistance to CO<sub>2</sub> assimilation by C<sub>3</sub> leaves (23), although there have been few studies concerned with the biochemistry of photosynthesis comparing wheat species differing in ploidy (6). The faster rates of photosynthesis achieved by C<sub>4</sub> plants (10) can be ascribed to the modified biochemistry and specialized leaf structure. Exper-

iments were therefore conducted to test whether a C<sub>4</sub> mechanism operates in any of several wheat genotypes known to have different photosynthetic rates.

RuBP<sup>2</sup> carboxylase is competitively inhibited by O<sub>2</sub>, and O<sub>2</sub> is also a competitive substrate leading to phosphoglycolate formation and photorespiration (20). In C<sub>4</sub> plants, these effects of O<sub>2</sub> on the RuBP carboxylase/oxygenase are decreased by a system that concentrates CO<sub>2</sub> in the cells containing this bifunctional enzyme, making the enzyme less sensitive to O<sub>2</sub> inhibition. Carboxylase of diploid and tetraploid wheats might be intrinsically less sensitive to O<sub>2</sub> than the enzyme in hexaploid varieties. Therefore, the oxygenase and carboxylase activities of purified enzyme from the several wheat genotypes were measured using concentrations of substrates reflecting those that might be expected in the chloroplast stroma.

## MATERIALS AND METHODS

**Plant Material.** Grains of *Triticum monococcum*(2X), *T. urartu*(2X), *T. dicoccum*(4X), and *T. dicoccoides*(4X) were kindly provided by R. B. Austin of the Plant Breeding Institute, Cambridge. Grains of *T. aestivum*(6X) cv Maris Dove and cv Maris Huntsman were purchased from the National Seed Development Organization Ltd., Cambridge, U.K.

For experiments involving photosynthesis by flag leaves in <sup>14</sup>CO<sub>2</sub>, seeds were surface sterilized and germinated on wet filter paper in the dark during 3 weeks at 0 to 5°C. At the end of April, 10 vernalized seedlings were planted per 8-inch pot in commercial compost outdoors in glass-covered cages. Exposures to <sup>14</sup>CO<sub>2</sub> were made as each species came to anthesis.

For studies of extracted RuBP carboxylase, seeds were sown in trays of compost, without vernalization, and grown for 3 weeks during summer in a glasshouse at 10 to 25°C.

**Photosynthesis in <sup>14</sup>CO<sub>2</sub>.** Selected pots of plants of each genotype were moved to a room at 23°C and illuminated with fluorescent and tungsten lamps giving a photon flux density of 400 μmol m<sup>-2</sup> s<sup>-1</sup> of photosynthetically active radiation. After at least 2 h, flag leaves were chosen at random and an area 1 × 1.5 cm of each was supplied for 10 s at 125 ml min<sup>-1</sup> with air containing <sup>14</sup>CO<sub>2</sub>, 320 μl l<sup>-1</sup> and 9.4 Ci mol<sup>-1</sup>, by means of a 'clamp-on' leaf chamber (24, 26). The leaf chamber was removed to allow further photosynthesis in ambient air for 5, 10, 20, 40, 60, or 120 s. A disc, 0.497 cm<sup>2</sup>, was then punched from the radioactive area of the leaf so that it fell directly into a tube containing liquid N<sub>2</sub> fastened below the punch. For each sample time, three separate leaves were supplied with <sup>14</sup>CO<sub>2</sub> and the three radioactive discs were collected together and extracted (2). Soluble products were separated by chromatography and their

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<sup>2</sup> Abbreviations: RuBP, D-ribulose 1,5-bisphosphate; v<sub>c</sub>, rate of carboxylation; v<sub>o</sub>, rate of oxygenation; PGA, 3-phospho-D-glyceric acid.

radioactivity determined (24, 26).

**Partial Purification of RuBP Carboxylase.** Plants from the glasshouse were kept overnight under a metal halide arc lamp giving a photon flux density at plant height of  $500 \mu\text{mol m}^{-2} \text{s}^{-1}$  (400–700 nm). The following morning samples of leaf material (7 g) were taken in the light and placed immediately in ice-cold water. At 0 to 5°C, the samples were cut with scissors into pieces 2 to 3 mm long and dropped into 25 ml of 20 mM Tris (pH 8.0), 10 mM  $\text{MgCl}_2$ , 10 mM  $\text{NaHCO}_3$ , 1 mM DTT, 1 mM EDTA with 0.002% w/v Hibitane (chlorohexidine diacetate) (buffer A). The suspension was homogenized using a Polytron PT20 Homogenizer (Northern Media Supply Ltd., Blanket Row, Hull) at a setting of 3.5 for 20 s and then for a further 10 s at the fastest speed that would allow steady circulation of the sample through the cutting head. The homogenate was filtered through a single layer of 50- $\mu\text{m}$  mesh nylon gauze and 20 ml of the filtrate were added to 4 g of solid  $(\text{NH}_4)_2\text{SO}_4$  in a 50-ml plastic centrifuge tube. The mixture was stirred mechanically with a glass spiral driven at 1200 rpm for 1 min to dissolve all the  $(\text{NH}_4)_2\text{SO}_4$  giving a solution that was 35% saturated with the salt. Leaf material from each genotype in turn was extracted until all the extracts were at this stage; this took a total of 90 min for the six extractions. The order in which genotypes were extracted was randomized and different for each of the three occasions when partial purifications were carried out. Precipitated protein was removed by centrifugation at 49,000g for 15 min. The supernatant liquids were decanted immediately into fresh tubes, each containing 2.4 g  $(\text{NH}_4)_2\text{SO}_4$ . Stirring as before was continued for 2 min so that all the  $(\text{NH}_4)_2\text{SO}_4$  dissolved to give 55% saturation. Precipitated protein was collected by centrifugation for 15 min at 49,000g and the supernatant liquids discarded. The protein fractions were each gently dissolved in 1 ml of 20 mM Tris (pH 8.0), 1 mM EDTA, 2 mM  $\text{MgCl}_2$ , 30 mM DTT, and 0.002% w/v Hibitane (buffer B), and the six solutions were each layered above linear sucrose density gradients 0.23 to 0.67 M sucrose in 28 ml in  $2.5 \times 8.5\text{-cm}$  tubes. These tubes were centrifuged for 2.5 h at 305,000g using a Beckman L2-65B ultracentrifuge with a 60 Ti Rotor. The contents of each tube were drawn off through the flow-cell of an LKB Uvicord II. The 15 ml of liquid from each sucrose gradient that contained most of the material absorbing light at 254 nm was collected and added to 4.9 g of  $(\text{NH}_4)_2\text{SO}_4$  in a 50-ml centrifuge tube with stirring as before but for 2 min. Protein recovered by centrifugation was dissolved in 0.5 ml of 119 mM Bicine (pH 8.2), 23.8 mM  $\text{MgCl}_2$  with 10 mM  $\text{NaHCO}_3$  (buffer C). Samples (0.4 ml) of each of the six protein solutions were desalted on columns of G-25 Sephadex (fine), previously equilibrated with buffer C, using the centrifugal technique of Helmerhorst and Stokes (13). The protein concentration in a sample from each desalted solution was estimated spectrophotometrically at 280 nm (22) and the concentration of protein in the remaining solution adjusted to  $25 \text{ mg ml}^{-1}$  by addition of buffer C. These solutions were stored either at 25°C or at 0°C during the period of subsequent assays.

**Measurement of Carboxylase and Oxygenase Activity.** Reaction mixtures at 25°C were prepared in the cell of an  $\text{O}_2$  electrode (Hansatech Ltd. Kings Lynn, U.K.) by first adding  $840 \mu\text{l}$  of  $\text{CO}_2$ -free 119 mM Bicine (pH 8.2), 23.8 mM  $\text{MgCl}_2$  (buffer D), after which the plug was fitted to limit the extent of contact with ambient air. Syringes were then used to add in succession  $20 \mu\text{l}$   $\text{NaH}^{14}\text{CO}_3$  (0.1 M,  $1.0 \text{ Ci mol}^{-1}$ ),  $10 \mu\text{l}$  carbonic anhydrase solution (25 units Sigma, from erythrocytes in buffer D [3]),  $10 \mu\text{l}$  40 mM RuBP and  $110 \mu\text{l}$  of  $\text{CO}_2$ -free water making adjustments of the plug as necessary. Reaction was initiated by adding  $10 \mu\text{l}$  of a solution of RuBP carboxylase and terminated after 60 s by injecting  $50 \mu\text{l}$  10 N HCOOH. During the 60-s reaction time,  $\text{O}_2$  uptake was measured with the recorder set to a sensitivity increased by 10 times and with approximately 90% of the

signal from the electrode 'backed off.' Samples of the acidified reaction mixture were evaporated to dryness and acid-stable radioactivity was measured by liquid scintillation counting. Individual enzymes were assayed as above in the same random order in which they were extracted. The assays were repeated three times in sequence in a total period of 2.5 h. After completion of the assays, protein in the partly purified preparations was determined colorimetrically (4). The partly purified enzymes were also examined by gel electrophoresis under conditions causing denaturation of the native protein (19).

## RESULTS

**Products of Photosynthesis in  $^{14}\text{CO}_2$ .** Figure 1 shows the amount of radioactivity in photosynthetic products in flag leaves of each of the six genotypes as a percentage of the total radioactivity extracted. Initially after exposure to  $^{14}\text{CO}_2$ , most  $^{14}\text{C}$  was present in phosphate esters, including PGA. The  $\text{C}_4$  acids, aspartate and malate, were scarcely radioactive. During the subsequent period of photosynthesis in  $^{12}\text{CO}_2$ ,  $^{14}\text{C}$  moved from the phosphate esters into sucrose, glycine, and serine. The  $^{14}\text{C}$  in  $\text{C}_4$  acids

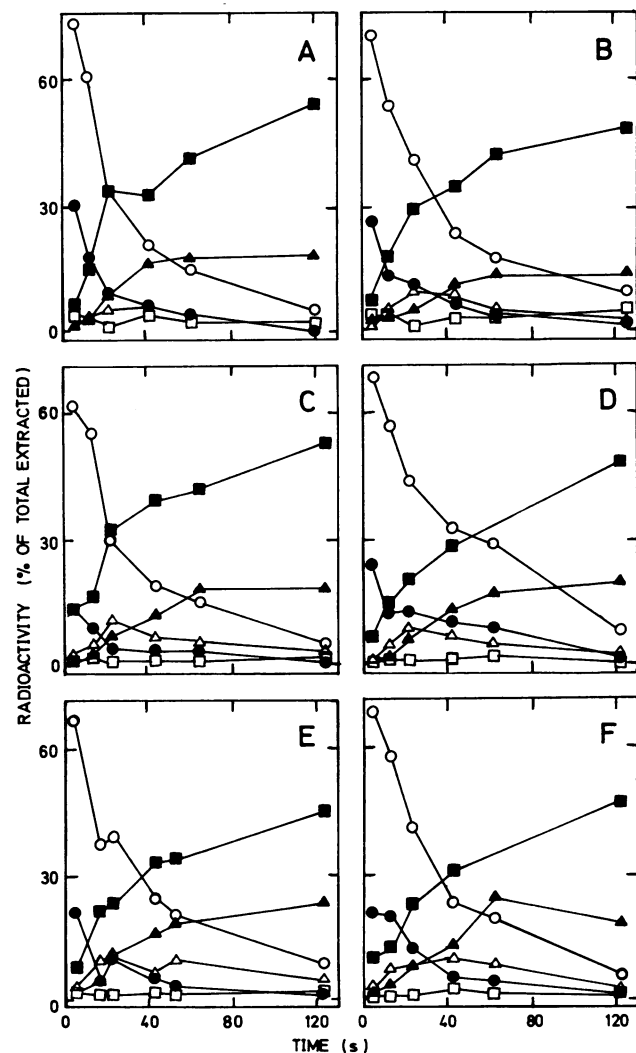


FIG. 1.  $^{14}\text{C}$  in photosynthetic intermediates after 10 s in air containing  $320 \mu\text{l/l}$   $^{14}\text{CO}_2$  ( $9.4 \text{ Ci/mol}$ ) by illuminated leaves of A, *T. arartu*(2X); B, *T. monococcum*(2X); C, *T. dicoccoides*(4X); D, *T. dicoccum*(4X); E, *T. aestivum*(6X) cv Maris Dove; F, *T. aestivum*(6X) cv Maris Huntsman. (○—○), Total phosphate esters; (●—●), PGA; (△—△), glycine; (▲—▲), serine; (■—■), sucrose; (□—□), aspartate and malate.

Table I. Carboxylase and Oxygenase Activities of Partially Purified RuBP Carboxylases from Wheat Genotypes

	<i>T. urartu</i> (2X)	<i>T. monococcum</i> (2X)	<i>T. dicoccoides</i> (4X)	<i>T. dicoccum</i> (4X)	<i>T. aestivum</i> (6X)	
					(Maris Huntsman)	(Maris Dove)
	<i>nmol min<sup>-1</sup> mg<sup>-1</sup> protein</i>					
Carboxylase ( $v_c$ )	491	482	567	551	503	527
Oxygenase ( $v_o$ )	80.2	78.5	95.9	94.9	83.8	90.3
$v_c:v_o$	6.18	6.30	6.03	5.98	6.06	6.09
Sources of Variation and Variance Ratio						
	Species	Replicates	Occasion			
Carboxylase ( $v_c$ )	130.1****	2.7*	47.7***			
Oxygenase ( $v_o$ )	9.5***	3.9**	92.6***			
$v_c:v_o$	1.0 (NS) <sup>b</sup>	12.1***	126.1***			

<sup>a</sup> (\*, \*\*, and \*\*\*), Significant differences at  $P = 0.05, 0.01, \text{ and } 0.001$ , respectively.

<sup>b</sup> NS, no significant differences at  $P = 0.05$ .

remained nearly constant at less than 5% of the total extracted  $^{14}\text{C}$  throughout the test period. After 120 s, the percentages of  $^{14}\text{C}$  in glycine plus serine were 18, 17.5, 22.5, 24, 29, and 21% for *T. urartu*(2X), *T. monococcum*(2X), *T. dicoccoides*(4X), *T. dicoccum*(4X), *T. aestivum*(6X) cv Maris Dove and cv Maris Huntsman, respectively. The corresponding percentages in sucrose were 55, 50, 54, 52, 46, and 48%. Thus, there appeared to be a lower incorporation into glycine and serine in diploid species. The experience of others, with a similar technique (18), suggest that standard errors are rather large and that the small differences observed above would need to be evaluated by further measurements before interpreting them in terms of relative rates of photorespiration. Furthermore, the amount of  $^{14}\text{C}$  present in glycine and serine at a given time is not necessarily an indication of total flux of assimilated carbon through the photorespiratory pathway; rather, it may reflect the size of metabolic pools and their relationship to one another.

**Properties of RuBP Carboxylase.** Gel electrophoresis in the presence of SDS showed that preparations of RuBP carboxylase from all six genotypes contained two main polypeptides corresponding to the large and small subunits of highly purified wheat carboxylase. The partially purified extracts also contained traces of at least two other proteins of larger molecular size than the large subunit of RuBP carboxylase. Immediately after completion of the purification procedure on each of the three occasions, assays for carboxylase and oxygenase activities were initiated. The mean results of triplicate measurements with three separate preparations of each enzyme are presented in Table I together with the results of evaluation by analysis of variance. Although there was considerable variation in specific activity of preparations between species for both carboxylase and oxygenase activities, the ratios  $v_c:v_o$  were not significantly different. There were significant occasion to occasion variations and variations between replicates on the same occasions, but the carboxylase and oxygenase activities in each species were similarly affected. The primary data showed that the measured value of  $v_c:v_o$  decreased with time after purification was completed whether the enzyme was stored at 0°C or 25°C  $v_c:v_o$ . Variation was probably the result of slight changes in the conditions of assay; slight changes in pH would result in large changes in the equilibrium of  $\text{HCO}_3^-$  with  $\text{CO}_2$  in solution. This could certainly explain the occasion to occasion variation when different preparations of buffer were involved: it is less easy to understand changes within an occasion.

## DISCUSSION

The results of the present experiments with  $^{14}\text{CO}_2$  agree with those of Champigny and Moise (6) in showing evidence of photorespiratory metabolism and no evidence for a  $\text{C}_4$  mechanism. This conclusion is also consistent with the measured values of photorespiration and  $\text{CO}_2$  compensation point in diploid, tetraploid, and hexaploid wheats (9). The slightly lower labeling of photorespiratory intermediates in the diploid and tetraploid genotypes, which need to be confirmed in further experiments, cannot be explained by differences in measured  $v_c:v_o$  ratios for the RuBP carboxylases. One alternative explanation is that the effective concentration of  $\text{CO}_2$  is higher in the stroma of diploids and tetraploids, compared to hexaploids, relative to the  $\text{O}_2$  concentration. Respired and photorespired  $\text{CO}_2$  may be produced in an especially favorable position relative to chloroplasts to allow re-assimilation, possibly amounting to an enrichment of  $\text{CO}_2$  near chloroplasts; such a situation has been postulated by Winter *et al.* (29) to explain the  $\text{C}_3/\text{C}_4$  intermediate character of *Moricandia arvensis*.

An extensive comparison of relative rates of RuBP carboxylation to oxygenation between species was made by Jordan and Ogren (16). Carboxylases from some photosynthetic bacteria had much lower  $v_c:v_o$  ratios than carboxylases of higher plants. There was also evidence of some small variations between higher plant genera. The present study suggests that within the species *Triticum* and among the particular genotypes studied, the variation in sensitivity to  $\text{O}_2$  is within the range of random error of present assay procedures. The variation in sensitivity to  $\text{O}_2$  of carboxylases from various species of higher plants was also shown to be small by less direct methods (4).

In the present study, the  $^{14}\text{C}$  was applied to flag leaves but a thorough study of the rate of net photosynthesis was not attempted. The carboxylases were extracted from young shoots and not the upper leaves of mature shoots. Although no specific claims have been made for a changing pattern of RuBP carboxylase isozymes during leaf ontogeny, there is evidence from isoelectric focusing of heterogeneity of the RuBP carboxylase subunit polypeptides in tobacco species (12).

There is evidence that high rates of photosynthesis require more RuBP carboxylase (14, 27). Leaves adapted to high light often have faster light-saturated rates of photosynthesis and a higher RuBP carboxylase content per unit leaf area than leaves grown at low light (5). In addition, where  $\text{CO}_2$  concentration is increased, as in  $\text{C}_4$  plants, less RuBP carboxylase is needed for

an equivalent rate of photosynthesis (25). It is therefore interesting that the concentration of RuBP carboxylase in the chloroplasts is less in a diploid than in a hexaploid wheat (7). This is more because of a decrease in chloroplast size with increase in ploidy than because of a change in RuBP carboxylase per plastid. No values (7) are given for RuBP carboxylase per unit leaf area but only per leaf section (5 mm long). It is clear that further understanding of the problem, if it depends on resistance to transfer of CO<sub>2</sub> to the RuBP carboxylase, requires a detailed study of the distribution of carboxylase within the chloroplast and of the chloroplast within a cell.

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