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Similarity of Ribulose-1,5-bisphosphate Carboxylases of Isogenic Diploid and Tetraploid Ryegrass (*Lolium perenne* L.) Cultivars[1]

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ABSTRACT

Partially purified ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) was isolated from diploid and tetraploid cultivars of ryegrass (*Lolium perenne* L.) using two separate methods. The apparent K_m (CO_2) values for the enzymes prepared by either method did not differ significantly between diploid and tetraploid when assayed by two separate techniques. The unpurified enzymes from freshly lysed (and fully functional) protoplasts of both diploid and tetraploid cultivars gave virtually identical apparent K_m (CO_2) values. There was no indication of large differences in affinity for CO_2 of illuminated intact protoplasts from the two cultivars.

Garrett (8) has observed differences in the affinity for CO_2 displayed by RuBP[4] carboxylase isolated from isogenic diploid and tetraploid forms of *Lolium perenne* L. The apparent K_m (CO_2) value for the diploid was 51 μM, which, in the light of current experience by other investigators, could be regarded as unusually high. That for the tetraploid was 22 μM, which could be regarded as more normal. Rathnam and Chollet (23) have reported that photosynthetic characteristics of illuminated protoplasts, including affinities for CO_2, differed for the two cultivars in a manner consistent with Garrett's findings.

The fact that the rate of photosynthesis by many higher plants is increased by a factor of 1.5 to 3 when the external CO_2 is increased from about 300 to about 1000 μl/l implies that photosynthetic carbon assimilation in air is often limited by the affinity of the carboxylase for CO_2. If this is so, a genetically controlled variation in the affinity of the carboxylase for CO_2 could be of considerable importance to plant breeders. However, RuBP carboxylase is easily inactivated *in vitro* and notoriously difficult to assay. Further verification of Garrett's findings, therefore, seemed to be an essential prerequisite to further investigation and this was undertaken at Rothamsted and Sheffield simultaneously. In a number of experiments in which isolation, activation, and assay conditions have been rigorously controlled, virtually identical K_m (CO_2) values have been recorded for RuBP carboxylases isolated from diploid and tetraploid material provided by Dr. M. K. Garrett (see below).

MATERIALS AND METHODS

PLANT MATERIAL

Tillers of the isogenic diploid (64038-50-308) and tetraploid (64038-1-312) cultivars of perennial ryegrass (*L. perenne* L.) were kindly supplied by Dr. M. K. Garrett, The Queens University of Belfast. At Sheffield, the plants were grown from November to February in pots in a glasshouse with supplementary lighting provided by metal-halide lamps giving 16-h days; the minimum temperature was 18 C. At Rothamsted, the plants were grown during December and January in pots in a glasshouse with supplementary lighting provided by mercury fluorescent lamps giving 16-h days; the minimum temperature was 10 C. The plants were propagated by vegetative means only. The ploidy of the plants was verified by chromosome counts.

PURIFICATION OF RuBP CARBOXYLASE

Method A. The method used at Sheffield was a modification of the method of A. Guest and R. J. Ellis (unpublished data). 20 g leaves were homogenized in 100 ml buffer A, which contained 50 mM Tris-HCl, 50 mM NaCl, 5 mM $MgCl_2$, and 2 mM EDTA (pH 7.5), to which 10 mM DTT and 0.5% (w/v) PVP were added immediately before use. The insoluble material was removed by centrifugation at 35,000*g* for 15 min and the soluble fraction was layered on stepped sucrose gradients. The gradients consisted of 2.5 ml each of the solutions 0.8, 0.6, 0.4, and 0.2 M sucrose in buffer A in 14-ml centrifuge tubes. The soluble fraction (2.5 ml) was layered onto each gradient. The gradients were centrifuged in an 8- × 14-ml fixed-angle rotor for 2.5 h at 65,000 rpm (250,000*g*) in an MSE Superspeed centrifuge. The RuBP carboxylase sedimented as a symmetrical peak to the level of the 0.6 M sucrose layer.

Method B. At Rothamsted, partly purified freeze-dried enzymes were prepared as described by Machler *et al.* (18) and Bird *et al.* (3).

ELECTROPHORESIS

Polyacrylamide gel electrophoresis was performed on 7.5% acrylamide gels using methods essentially as described by Davis (5) and Ornstein (19). Protein (200 μg) was applied to each gel and electrophoresis was conducted at 1 mamp/tube until the marker dye had been carried out of the gel (about 4 h). Gels were stained with Coomassie brilliant blue. Gel electrophoresis in the

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[4] Abbreviations: RuBP, ribulose 1,5-bisphosphate; Bicine, *N,N'*-bis(2-hydroxyethyl)glycine.

presence of SDS for the determination of subunit molecular weights was performed as described by Weber and Osborn (28).

ACTIVATION OF PURIFIED ENZYMES

RuBP carboxylase purified by Method A was activated by incubation with 20 mM $MgCl_2$, 10 mM $NaHCO_3$, and 5 mM DTT (pH 7.9) for 10 min at 20 C in a total volume of 100 μl. After activation, 40 μl (18 to 25 μg protein) were removed for assay.

Freeze-dried powders obtained by Method B were dissolved in water and the solution was mixed with an equal volume of 200 mM Bicine, 40 mM $MgCl_2$, 20 mM $NaHCO_3$, and 2 mM DTT to give a final protein concentration of 5 mg/ml. The solution was kept at 25 C for 5 h to activate the enzyme; prolonged activation is necessary for RuBP carboxylase that has been crystallized or kept at low temperature (18).

ISOLATION OF PROTOPLASTS

Protoplasts were isolated essentially by the method of Edwards *et al.* (7). Young, fully expanded leaves (10 g) were sliced by hand with a razor blade into strips 0.5 to 1 mm wide. The strips were incubated in the light for 3 h at 28 C in 50 ml enzyme medium containing 0.5 M sorbitol, 1 mM $CaCl_2$, 0.5% (w/v) BSA, 3% (w/v) cellulase (Onozuka 3S), 0.5% (w/v) pectinase (Macerozyme R10), and 5 mM Mes (adjusted to pH 5.5). After incubation, the enzyme medium was removed and the tissue was washed three times with 20-ml aliquots of 0.5 sorbitol, 1 mM $CaCl_2$, 5 mM Mes (pH 6.0). The protoplasts released were collected by centrifugation and purified on a sucrose-sorbitol step gradient as described by Edwards *et al.* (7). Purified protoplasts were stored on ice in 0.5 M sucrose plus sorbitol, 1 mM $CaCl_2$, 5 mM Mes (pH 6.0). Yields of protoplasts from the diploid cultivar were consistently lower (200 to 500 μg Chl) than yields from the tetraploid cultivar (>2 mg Chl). Rates of CO_2-dependent O_2 evolution by protoplasts from both cultivars were similar, the highest recorded with diploid protoplasts being 98 μmol O_2 mg^{-1} Chl h^{-1} and that with tetraploid protoplasts being 109 μmol O_2 mg^{-1} Chl h^{-1}.

ASSAY OF PHOTOSYNTHESIS

CO_2-dependent O_2 evolution by protoplasts was followed polarographically at 20 C in a twin O_2 electrode system (6) purchased from Hansatech Ltd., Hardwick Industrial Estate, King's Lynn, Norfolk. Photosynthesis was measured in a medium containing 0.5 M sorbitol, 1 mM $CaCl_2$, 20 mM Tricine (pH 7.6), and $NaHCO_3$ in varying concentrations. For assay of RuBP carboxylase from illuminated protoplasts, aliquots equivalent to 5 μg Chl were removed from the sample in the O_2 electrode after the maximum rate of O_2 evolution had been achieved. This was usually 4 to 5 min after the commencement of illumination. For assay of RuBP carboxylase from nonilluminated protoplasts, aliquots were removed from a suspension in the assay medium after preincubation for 4 min in the dark.

ACTIVATION OF PROTOPLAST EXTRACT

Complete activation was achieved by lysing a concentrated suspension of protoplasts in 20 mM $MgCl_2$, 10 mM $NaHCO_3$, and 5 mM DTT (pH 7.9) by addition of 0.25% (w/v) Triton X-100 followed by incubation for 10 min at 20 C. After activation, an aliquot containing 5 μg Chl was removed for addition to the RuBP carboxylase assay medium.

ASSAY OF RuBP CARBOXYLASE

The procedure for enzyme prepared by Method A or released from protoplasts was based on the spectrophotometric assay of Lilley and Walker (12). All solutions were rendered CO_2- and O_2-free by using boiled deionized H_2O which was flushed with and stored under N_2. All solutions were flushed again with N_2 immediately prior to use in the assay. The pH of buffers was adjusted with CO_2-free KOH (KOH pellets rinsed three times with CO_2-free water, dissolved in CO_2-free water, and flushed with N_2).

The assay mixture contained 50 mM Hepes-KOH, 10 mM KCl, 1 mM EDTA, 15 mM $MgCl_2$, 5 mM DTT, 5 mM phosphocreatine, 5 mM ATP, 2 units creatine phosphokinase (EC 2.7.3.2), 5 units P-glycerate kinase (EC 2.7.2.3), 5 units glyceraldehyde-3-P dehydrogenase (EC 1.2.1.12), 0.26 mM NADH, and $NaHCO_3$ in varying concentrations (pH 7.9). Due allowance was made for the bicarbonate transferred with protoplasts, activated protoplast extract, and activated purified enzyme. Immediately prior to assay, activated purified enzyme, protoplast extract, or protoplasts were added to the cuvette and the reaction was initiated by the addition of 0.5 mM RuBP. The final reaction volume was 1 ml and the cuvettes were fitted with stoppers which reached to the surface of the liquid so that there was no gas phase present. The reaction was followed at 20 C by recording the decrease in A_{340} due to oxidation of NADH. For determination of apparent $K_m(CO_2)$ or where concentrations of $NaHCO_3$ less than saturating were used, 25 units carbonic anhydrase (EC 4.2.1.1) were incubated in each reaction mixture (3). When the enzyme to be assayed was from intact protoplasts, 0.25% (w/v) Triton X-100 was included in each assay mixture to ensure rapid lysis of the protoplasts (25). RuBP was dissolved immediately before use to minimize formation of xylulose-5-bisphosphate, a potent inhibitor of RuBP carboxylase (20).

Carboxylation by activated enzyme from freeze-dried preparations (Method B) was measured by procedures similar to those described by Lorimer *et al.* (15). Reaction mixtures were prepared in screw-cap septum vials supplied by Pierce and Warriner Ltd., Chester, United Kingdom. (Catalog No. 13109 and 12712, respectively, for vials and septa). The mean total internal volume of 12 such vials was 4.96 cm^3, with a range from 4.87 to 5.07 cm^3. To each vial was added a miniature glass-covered iron stirring bar to be driven from the outside by a magnetic stirrer immersed in a constant-temperature water bath. To each vial then was added 10 μl 20 mM RuBP. The vials then were closed and gassed for 1 min with pure N_2 or CO_2-free air, at 125 ml min^{-1}, through hypodermic needles inserted through the septa. While gassing was continued, 0.42 ml 119 mM Bicine buffer (pH 8.2), 23.8 mM $MgCl_2$ was added. This buffer had been previously equilibrated with pure N_2 or with CO_2-free air (15), as appropriate. Gassing was stopped by removing the needles, and the required amount of $NaH^{14}CO_3$ was added in 0.05 ml to give a total, after addition of the carboxylase, of from 0.295 to 2.5 μmol HCO_3. The vials then were incubated for more than 1 h at 25 C while an equilibrium was established between CO_2 in the gas phase, CO_2 in solution, and HCO_3^- in solution. Reaction was started by adding 10 μl of a solution of carbonic anhydrase in 10 mM Bicine (pH 8.2), 1 mM EDTA (3) to give 25 units/vial (24, 29), followed by the rapid addition of 10 μl of the solution of activated carboxylase (50 μg protein with 0.1 μmol $NaHCO_3$). The contents of the vial were stirred during, and for 3 s after, addition of the carboxylase. The period of the reaction was timed from the addition of this enzyme. Reaction was terminated after 1 min by rapidly injecting 0.25 ml 4 N HCl followed immediately by stirring for 10 s. Contents of the septum vials were transferred quantitatively to vials for liquid scintillation counting and evaporated to dryness overnight at 100 C. Residues were dissolved in 1 ml water, and this solution was mixed with 10 ml scintillant (22). From the ^{14}C content of the residue, the rate of carboxylation was calculated for each of the different amounts of added $NaHCO_3$.

PROTEIN DETERMINATIONS

Protein was determined by the method of Lowry *et al.* (16) or by the method of Markwell *et al.* (17) following precipitation by the method of Peterson (21).

CALCULATION OF RESULTS

Rates of carboxylation at different bicarbonate/CO_2 concentrations were analyzed using the statistical method of Wilkinson (30). The concentration of dissolved CO_2, the substrate used by the RuBP carboxylase (4), was calculated from pH and the amount of bicarbonate added using the Henderson-Hasselbalch equation. Like Garrett (8), and unlike Bird *et al.* (3), we used the data of Harned and Bonner (9) for pK_1' for H_2CO_3 at an ionic strength equivalent to that of the assay mixture. The solubility coefficient for CO_2 in the reaction mixture was extrapolated from the data of Harned and Davis (10). In this way, we calculate that, at 25 C in the assay by $^{14}CO_2$ fixation, the ratio for CO_2 in solution to CO_2 in the gas phase to bicarbonate in solution is 1:11.2:120.2, *i.e.* the CO_2 in solution is only 0.755% of the added bicarbonate. For the spectrophotometric assay, the ratio of CO_2 to bicarbonate in solution is 1:63.1, *i.e.* CO_2 in solution is 1.56% of the added bicarbonate. Here, there is no gas phase to be considered.

Because of the variety and complexity of the media used in assays of RuBP carboxylase, it is important that authors should adopt a consistent policy with regard to the value of pK_1' for carbonic acid used to calculate free CO_2 in solution or that they give enough information to allow proper comparison between values they obtain and others that are published. Thus, in the spectrophotometric assay used above, one might use a value of 6.39 (the pK_1 at infinite dilution), instead of 6.10 for $\mu = 0.175$ extrapolated from the data of Harned and Bonner (9); the former would suggest 3% of the added bicarbonate would dissociate to free CO_2 in solution; the latter would suggest 1.56%. This would give a factor of almost 2 between calculated values of $K_m(CO_2)$. No doubt the pK_1' and solubility of CO_2 should be measured for each particular assay medium employed inasmuch as the ionic and nonionic species present do not have exactly the same effect as NaCl employed by Harned and Bonner (9) and Harned and Davis (10). One could also argue that the $K_m(CO_2)$ for the enzyme will also be affected by the solutes present and that estimated values may have little relevance to the kinetic properties of the enzyme *in vivo*.

Table I. *Kinetic Constants of Purified RuBP Carboxylase Isolated from Diploid and Tetraploid Ryegrass Cultivars by Method A*

Results are means of three different experiments. Values in parentheses are the SE.

Cultivar	Apparent K_m ($NaHCO_3$)	Apparent K_m (CO_2)	V_{max}
	mM	μM	μmol mg^{-1} protein min^{-1}
Diploid	0.63 (±0.07)	9.8 (±1.1)	0.68 (±0.08)
Tetraploid	0.58 (±0.07)	9.1 (±1.0)	0.59 (±0.09)

RESULTS

Comparison of Enzymes by Electrophoresis. From the results of polyacrylamide gel electrophoresis, the purified RuBP carboxylase from the both ryegrass cultivars consisted largely of a single protein with few minor contaminants. The mobility of the main band was identical for both the diploid and tetraploid cultivars. When the purified enzymes were treated with SDS and subjected to disc-gel electrophoresis (26, 28), the major band observed in detergent-free gel was absent and two other major bands were evident. These bands were estimated to be of mol wt 52,500 and 18,000. Similar values were obtained with both the diploid and tetraploid ryegrasses and are consistent with those previously published for the subunits of RuBP carboxylase (1).

Kinetic Constants of Enzymes from Diploid and Tetraploid Cultivars. The conditions for activation of enzyme purified by Method A were carefully examined as it was essential for the accurate determination of K_m and V_{max} that the enzymes were activated fully. The enzymes from the diploid and tetraploid cultivars were not found to differ in requirements for activation. In the spectrophotometric assay, there was a very short initial lag in the oxidation of NADH, similar to that seen with chloroplast extracts (12). The reaction then was linear for at least 1.5 min. In agreement with work carried out at Rothamsted (3), it was found that carbonic anhydrase stimulated the amount of CO_2 fixed at low levels of $NaHCO_3$ and in the absence of carbonic anhydrase the apparent K_m for CO_2 was increased. Carbonic anhydrase was therefore normally included in assay mixtures.

Table I shows kinetic constants for enzymes purified from the diploid and tetraploid cultivars by Method A, assayed by the spectrophotometric method. Table II shows similar data for enzymes purified by Method B and assayed by the $^{14}CO_2$ fixation technique. We stress that the experiments leading to the data in Tables I and II were performed in different laboratories by different researchers. The differences in the absolute values between the two tables may be explained by differences in the conditions of assay. Even so, in each set of conditions, the K_m for CO_2 of enzymes from diploid and tetraploid ryegrass are not significantly different when measured in the presence of N_2. Table II also shows that apparent K_m values do not differ significantly between enzymes from diploid and tetraploid cultivars when measured in the presence of air, although the values in air are always greater than in N_2 because of the competitive inhibition by O_2. Values for the apparent K_m (CO_2) in N_2 in both Tables I and II are lower than those reported by Garrett (8). Values for V_{max} are somewhat higher than those reported by Garrett (8). We do not attach much importance to the slightly higher V_{max} values found for enzyme from diploid as compared with tetraploid ryegrass (Tables I and II); this may be due to differences in purity

Table II. *Kinetic Constants of RuBP Carboxylase Isolated from Diploid and Tetraploid Ryegrass by Method B*

Values in parentheses are the SE.

Experiment	Apparent K_m (CO_2)				V_{max}			
	Diploid		Tetraploid		Diploid		Tetraploid	
	N_2	Air	N_2	Air	N_2	Air	N_2	Air
	μM				μmol CO_2 fixed mg^{-1} protein min^{-1}			
A	14.1 (0.7)	19.6 (0.6)	13.9 (0.1)	20.0 (0.5)	0.91 (0.02)	0.94 (0.01)	0.71 (0.02)	0.79 (0.01)
B	14.4 (0.4)	15.6 (1.2)	13.5 (0.3)	18.1 (0.5)	0.91 (0.01)	0.74 (0.03)	0.76 (0.01)	0.72 (0.01)
C	14.3 (0.2)	22.0 (0.9)			0.90 (0.01)	1.05 (0.02)		
Mean K_i (O_2) mM	0.77		0.67					

Table III. *Properties of RuBP Carboxylase in Protoplasts and Activated Protoplast Extract from Diploid and Tetraploid Ryegrass Cultivars*

Intact protoplasts were incubated in 0.5 M sorbitol, 1 mM $CaCl_2$, 20 mM Tricine (pH 7.6), and 2 mM $NaHCO_3$. RuBP carboxylase was measured after 4 min illumination when O_2 evolution was at its maximum rate. Activated protoplast extract was prepared by incubating lysed protoplasts with 20 mM $MgCl_2$, 10 mM $NaHCO_3$, and 5 mM DTT for 10 min at 20 C at pH 7.9. In all cases, 5 μg Chl was removed for assay of RuBP carboxylase. Results are means of two independent experiments. Values in parentheses are the SE.

Enzyme Source	Cultivar	Apparent K_m ($NaHCO_3$)	Apparent K_m (CO_2)	V_{max}
		mM	*μM*	*μmol mg^{-1} Chl h^{-1}*
Illuminated protoplasts	Diploid	0.83 (±0.07)	12.9 (±1.0)	378 (±31)
	Tetraploid	0.93 (±0.10)	14.5 (±1.6)	337 (±45)
Activated protoplast extract	Diploid	0.64 (±0.04)	10.0 (±0.6)	360 (±36)
	Tetraploid	0.72 (±0.07)	11.2 (±1.1)	348 (±18)

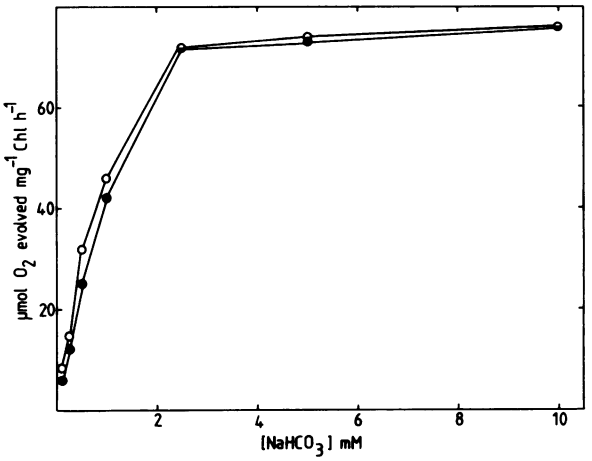

FIG. 1. O_2 evolution by diploid (○) and tetraploid (●) ryegrass protoplasts as a function of bicarbonate concentration. Protoplasts were illuminated in 0.5 M sorbitol, 1 mM $CaCl_2$, 20 mM Tricine (pH 7.6), and $NaHCO_3$, as shown.

or to differences in extent of irreversible inactivation during purification. Especially for measurements made in N_2, the values of K_m in Tables I and II are reproducible from occasion to occasion. Also, standard errors are small so that we can exclude as highly improbable that differences in the K_m for carboxylase from diploid compared to tetraploid ryegrasses are as large as those reported by Garrett (8). We concede that there are small differences in temperature and pH of the assay procedures used by us compared to those used by Garrett (8): pH 7.9 and 20 C in the spectrophotometric assay and pH 8.2 and 25 C in the $^{14}CO_2$ fixation assay compared to pH 8.1 and 25 C. Also, we fully appreciate that the circumstances controlling activation of the enzyme may not yet be fully understood. Nevertheless, by keeping conditions as similar as possible during preparation and assay, it seems that the carboxylases from the two sources have very similar properties, as may have been expected from the description "isogenic."

Affinity for CO_2 and RuBP Carboxylase Freshly Released from Protoplasts. When intact protoplasts were added to the RuBP carboxylase assay mixture, the absorbance changes in the spectrophotometric assay were similar to those obtained with the purified enzyme, provided sufficient Triton X-100 was included in the assay mixture to cause rapid lysis. No inhibitory effect of Triton X-100 was observed, but a longer initial lag or period of nonlinear CO_2 fixation occurred at Triton X-100 concentrations below 0.2%, indicating a relatively slow lysis of the protoplasts. The linearity of the CO_2 fixation observed indicated that the activation state of the carboxylase did not markedly change during the 1.5-min period of the assay. The carboxylase activity released from protoplasts in the presence of 2 mM $NaHCO_3$ was greater than the maximum rate of CO_2-dependent O_2 evolution in illuminated protoplasts (Fig. 1).

The activity of the enzyme released from protoplasts was increased in the presence of $MgCl_2$, $NaHCO_3$, and DTT and the rate of this activation was similar to that for the purified enzyme. The enzyme was about 70% activated in the illuminated protoplasts. This contrasts somewhat with experiments reported with wheat protoplasts (25), in which nearer 100% of the RuBP carboxylase from protoplasts preincubated in the light was in a fully active state, but agrees with results published for spinach chloroplasts (2) and with results obtained with spinach protoplasts and chloroplasts and pea chloroplasts (unpublished data). Values obtained for the activities of RuBP carboxylase from protoplasts or activated protoplast extracts were similar in both diploid and tetraploid ryegrass cultivars.

The values for apparent K_m and V_{max} for the RuBP carboxylase, released directly into the assay mixture from illuminated protoplasts and in fully activated protoplast extract, are given for both cultivars in Table III. Apparent K_m (CO_2) values were similar for diploid and tetraploid protoplasts, although the K_m (CO_2) values obtained were somewhat lower for the activated protoplast extract than for enzyme freshly released from illuminated protoplasts.

Photosynthesis by Protoplasts. Protoplasts were isolated from both the diploid and tetraploid cultivars of ryegrass. The rates of CO_2-dependent O_2 evolution by protoplasts at different concentrations of $NaHCO_3$ are shown in Figure 1. The results are similar with protoplasts from both cultivars. It is not possible to obtain a linear double-reciprocal plot from these data, presumably because electron transport becomes limiting at high CO_2 concentrations as it does in spinach chloroplasts (13, 27). Thus, the relationship between bicarbonate concentration and activity of the protoplasts is similar to that between bicarbonate concentration and activity of intact chloroplasts and does not allow the application of conventional kinetic analysis as used in enzyme studies (11, 13). It is possible to determine the concentration of bicarbonate which supports 50% of the maximum measured rate of photosynthesis by the protoplasts. For the diploid ryegrass, this concentration was 0.77 mM $NaHCO_3$ and, for the tetraploid ryegrass, it was 0.85 mM $NaHCO_3$. At pH 7.6 and 20 C, these are equivalent to 44.0 and 48.6 μM CO_2, respectively, and are similar to values reported with intact spinach chloroplasts (13).

DISCUSSION

Under the conditions used at Sheffield and Rothamsted, the enzymes reported by Garrett to have a 2-fold difference in affinity for CO_2 gave virtually identical K_m (CO_2) values. The difficulties associated with measuring RuBP carboxylase have recently led both groups to modify existing techniques. For this reason, full

details are included wherever these are known to be important. The results presented have been evaluated by the statistical treatment of Wilkinson (30); this relieves the investigator of the onus of deciding the line to be fitted to points on the double-reciprocal plot and provides a measure of the accuracy of extrapolated values for kinetic constants. The low standard errors obtained validate the assay methods employed. With the data obtained, there is no difficulty in fitting lines by eye to the double-reciprocal plots and such a method would not have produced a different conclusion. Furthermore, studies with unpurified enzyme from freshly lysed (and fully functional) protoplasts confirm results obtained with the purified enzymes. It was not possible to provide an accurate measure of affinity for CO_2 of illuminated protoplasts, but there was no indication of large differences between protoplasts from the two ryegrass cultivars.

RuBP carboxylase was long thought to be unequal to its task *in vivo* because of its seemingly low affinity for CO_2. The work of Lorimer *et al.* (14) on the activation of the purified enzyme did much to resolve this old enigma. There remains some mystery concerning full activation of the enzyme following different storage conditions, clarification of which, together with an explanation of the discrepancy between our results and those of Garrett (8), awaits further investigation.

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