Photosynthetic and Photorespiratory Carbon Metabolism in Mesophyll Protoplasts and Chloroplasts Isolated from Isogenic Diploid and Tetraploid Cultivars of Ryegrass (*Lolium perenne* L.)

C. K. M. Rathnam and Raymond Chollet

Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln, Nebraska 68583

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**ABSTRACT**

Photosynthetic 14CO2 fixation, 14Cglycolate formation, and the decarboxylation of [1-14C]glycolate and [1-14C]glycine by leaf mesophyll protoplasts isolated from isogenic diploid and tetraploid cultivars of ryegrass (*Lolium perenne* L.) were examined. The per cent O2 inhibition of photosynthesis in protoplasts from the tetraploid cultivar was less than that of the diploid line at both 21 and 49% O2. Kinetic studies revealed that the Km (CO2) for photosynthesis by the diploid protoplasts was about twice that of the tetraploid line. In contrast, the Km (O2) for protoplast photosynthesis was similar in both cultivars, as was the potential for oxidizing glycoglate and glycine to CO2 via the photorespiratory carbon oxidation cycle. Although the maximal rates of glycoglate accumulation by the isolated protoplasts in the presence of 21% O2 and a glycoglate oxidase inhibitor were similar in the two cultivars, the percentage of total fixed 14C entering the 14Cglycolate pool and the ratio of the rate of 14Cglycolate formation to 14CO2 fixation at 21% O2 and low pCO2 were about two times greater in protoplasts and intact chloroplasts isolated from the diploid line compared to the tetraploid. These results fully support the recent observation that a doubling of ploidy in various ryegrass cultivars reduced the Km (CO2) of purified ribulose bisphosphate carboxylase-oxygenase by about one-half without affecting the Km (O2) (Garrett 1978 Nature 274: 913-915).

This laboratory has a continuing interest in studying photosynthetic carbon metabolism in higher plants with altered photorespiration and the associated O2 inhibition of photosynthesis (7, 20--22). Recently, Garrett (11) reported a ploidy effect on the Km (CO2) of Rbu-P2 4-carboxylase-oxygenase (EC 4.1.1.39) purified from various cultivars of ryegrass, including an isogenic diploid and tetraploid line. Whereas the Km (CO2) (carboxylase) and Km (CO2) (oxygenase) for the CO2/Mg2+-activated enzyme from the diploid (2X) cultivars were about twice those from the tetraploid (4X) lines, the kinetic constants for O2 were identical for the two groups. Correlative leaf CO2 exchange studies indicated that photorespiration, estimated by the CO2 compensation concentration and the postillumination CO2 burst, was also reduced in the tetraploid cultivars. It seems that increased ploidy decreases photorespiration in ryegrass cultivars and that, mechanistically, this is correlated with an increased affinity of Rbu-P2 carboxylase-oxygenase for substrate CO2.

These investigations were undertaken to elucidate any differences in photosynthetic and photorespiratory carbon metabolism in leaf slices, mesophyll protoplasts and chloroplasts isolated from the isogenic diploid and tetraploid lines of ryegrass. Our results fully support Garrett's observation (11) that ploidy level affects photorespiration in ryegrass and that this genetically controlled variation in photorespiration relative to photosynthesis is correlated with the kinetic properties of photosynthetic CO2 fixation catalyzed by Rbu-P2 carboxylase.

**MATERIALS AND METHODS**

**Plant Material.** The isogenic diploid (64038-50-308) and tetraploid (64038-1-312) cultivars of perennial ryegrass (*Lolium perenne* L.) used in the present study were originally derived by repeated cloning of a mixoploid line detected among colchicine-treated 2X seedlings (1). Seeds and tillers of this isogenic material were kindly supplied by M. K. Garrett, The Queen's University of Belfast. The plants were grown in Metro-Mix potting medium in a controlled environment room (800 μE m⁻² s⁻¹, 16-h photoperiod, 21C day/16C night), watered daily, and nutrients were supplied twice weekly in the form of a modified Hoagland solution.

**Preparation of Leaf Slices, Mesophyll Protoplasts, and Chloroplasts.** Young, fully expanded leaves were mechanically sliced into 0.5-mm-wide sections (18), suspended in 0.3 m sorbitol containing 50 mM Hepes, 1 mM MgCl2, 1 mM MnCl2, 2 mM KH2PO4, and 3 mM isosorbate adjusted to pH 7.6 (medium A), and maintained at room temperature. Leaf slices (2-3 g) were incubated for 2 h at 30 C in 30 ml of digestion medium containing 0.5 m sorbitol, 5 mM Mes, 1 mM CaCl2, 0.05% (w/v) BSA, 2% (w/v) Onozuka R-10 cellulase, and 0.3% (w/v) Macerozyme pectinase (both from Yakult Biochemicals Co., Nishinomiya, Japan) adjusted to pH 5.5 with KOH. Released mesophyll protoplasts were collected and subsequently purified on a sucrose-sorbitol step gradient as described by Edwards et al. (10), and finally stored on ice in medium A. Intact chloroplasts were isolated by gentle protoplast rupture as described by Rathnam and Edwards (23) and were pelleted by centrifugation at 270g for 90 s. The chloroplasts were isolated and resuspended in medium A containing 0.1 mM KH2PO4 and stored on ice.

**Assay Conditions.** 14CO2 fixation, [1-14C]glycine decarboxylation and [1-14C]glycolate synthesis assays were performed in serum-stoppered glass vials containing a standard CO2-less reaction mixture of 0.3 m sorbitol, 50 mM Tricine, 1 mM MgCl2, 1 mM MnCl2, 2 mM KH2PO4 (0.1 m with chloroplasts), and 3 mM isosorbate adjusted to pH 8.0 (20, 21). Other additions were as indicated for the individual experiments. The assays were run in a constant temperature water bath maintained at 30 C; illumination was provided by sodium discharge lamps.
were or 13.5 removed contents of carrier H2O were indicated, nated for reaction vials were preincubated at 21% O2 in the presence of 0.05 ml 50% (w/v) trichloroacetic acid or 4 n acetic acid. The vials were flushed with air to ensure complete removal of unfixed 14CO2 and dpm-determined by liquid scintillation spectroscopy (20, 21).

Glycogen and Glycine Decarboxylation. Decarboxylation of glycogen and glycine at the C-1 position by isolated protoplasts (13-40 μg Chl) in light and darkness was followed directly using 5 mM [1-14C]glycogen (0.1 Ci/mol) (Amersham/Searle) and 5 mM [1-14C]glycine (0.1 Ci/ml) (Amersham/Searle) as previously described (19, 21). The protoplasts were preincubated at 21% O2 in the presence of 0.05 ml of various enzyme inhibitors (adjusted to pH 8.0 with KOH) for 20-30 min at 25°C before the assays were initiated by injecting the 1.4C-substrate. All values were corrected for nonenzymic decarboxylation with boiled protoplast controls.

Glycate Synthesis. Photosynthetic [14C]glycogen formation by isolated protoplasts and chloroplasts was followed using NaH14CO3 or [U-14C]riboside-5-P (Schwarz/Mann). [U-14C]Ribose-5-P was further purified by successive one-dimensional ascending chromatography on Whatman 3MM filter paper in acetone-30% (v/v) acetic acid (1:1, v/v), and n-amyl alcohol-5 n formic acid (1:1, v/v). Between the first and second solvent systems and after the final run, the chromatograms were air-dried, the area containing ribose-5-P eluted with H2O, lyophilized, and redissolved in a small amount of water (6).

The assay conditions for photosynthetic [14C]glycogen formation were identical to those described above for 14CO2 fixation except that the protoplast assay medium routinely contained 1 mM HBA (adjusted to pH 8.0 with KOH) to inhibit completely the subsequent metabolism of glycogen (see Fig. 2). The reactions were initiated by injecting NaH14CO3 (0.1-5.0 mM final concentration) (2.5 Ci/mol) or repurified [U-14C]riboside-5-P (final concentration, 0.5 mM; 0.3 Ci/mol) and terminated after 10 min by injecting 0.25 ml 4 n acetic acid. The acidified reaction vials were unsealed, purged with air to remove unfixd 14CO2, and the contents neutralized with 0.2 ml 4 n NaOH. Two 50-μl aliquots were removed from the [14C]carbonate-initiated samples to determine the rate of 14CO2 fixation during the 10-min reaction interval.

To the pooled contents of three replicate reaction vials, 2 μmol of carrier glycogen was added and the contents were extracted successively with 5-ml aliquots of boiling 80% (v/v) ethanol, 50% (v/v) ethanol, and H2O. The extracts were pooled, cooled, and centrifuged at 20,000g for 30 min. The supernatant was decanted and the pellet was washed three times with 5 ml H2O. The ethanol/water-soluble supernatant fractions were pooled and fractionated on a Bio-Rad AG 1-X8 column (acetate; 200-400 mesh) (1 x 6.5 or 13.5 cm). The column was washed with 30 ml H2O and 30 ml 1 n acetic acid, and the glycogcn-glycerate fraction was eluted with 30 ml 4 n acetic acid (6). The eluant was taken to dryness at 30°C under reduced pressure and residual acetic acid vapors were removed under a stream of N2. The residue was redisolved in H2O, spotted on precoated TLC plates of Cellogel MN 300 using the organic phase of n-amyl alcohol-5 n formic acid (1:1, v/v) as the solvent (6). The developed chromatograms were immediately sprayed with 1 mM NaHCO3 to prevent volatilization of glycolic acid, air-dried, and scanned for radioactivity. The radioactive peak which co-chromatographed with authentic glycogen was eluted and dpm determined by liquid scintillation spectroscopy (6, 21). Recovery of [1-14C]glycogen internal standard was always greater than 90%. All values were corrected for boiled protoplasm or chloroplast controls run under identical conditions. The TLC separation of glycogen described above is superior to other published procedures (cf., ref. 16) in that development of the 20-cm plates is complete within 2-3 h and, more important, glycogen is well resolved (Rf=0.49) from glyceraldehyde (Rf=0.20) which coelutes from the acetate column.

Inhibitors. The enzyme inhibitors used in the present study were: maleate for PEP carboxylase (18, 20), D,L-glyceraldehyde for the C3 photosynthetic carbon reduction cycle (26), HBA for glycogen oxidase (13, 24), and INH for glycine decarboxylase (3, 24). HBA was a generous gift from J. C. Servaites.

Chl was estimated as described previously (20). O2 and CO2 solubilities were calculated from standard solubility tables (20).

RESULTS AND DISCUSSION

Assay of crude leaf extracts from the isogenic 2x and 4x cultivars for various enzymes associated with C3 and C4 photosynthesis indicated that the activity of RuBp-P2 carboxylase on a Chl basis was similar in both lines (592 to 618 μmol/mg Chl-h). The presence of a C3-like PEP carboxylase activity (15 to 21 μmol/mg Chl-h compared to 27 μmol/mg Chl-h in a C3 Panicum species (20)) and no detectable activity of pyruvate Pi dikinase, the most diagnostic marker enzyme for C4 photosynthesis (22), suggest the absence of even a limited C4-type photosynthesis in these cultivars.

O2 Inhibition of Photosynthesis. Table I shows the effect of pO2 on photosynthesis by isolated leaf mesophyll protoplasts at low HCO3 (0.48 mM, 10.5 μM CO2 at pH 8.0). The rate of 14CO2 fixation by the 4X protoplasts was 28, 47, and 83% greater than the 2X line at 21%, and 49% O2, respectively. The per cent O2 inhibition of photosynthesis in the tetraploid cultivar was significantly less than that observed with the diploid line at both 21% and 49% O2. Similar experiments with mechanically prepared thin leaf slices further documented the differing O2 sensitivity of photosynthesis by the 2X and 4X cultivars (data not shown). Maleate, a specific inhibitor of PEP carboxylase under the experimental conditions employed (18, 20), had no effect on either the extent of O2 inhibition or the rate of photosynthesis by thin leaf slices (data not shown) or mesophyll protoplasts (Table I) isolated from the diploid and tetraploid lines. These observations, together with the in vitro enzyme data discussed above, indicate a lack of PEP carboxylase involvement in photosynthetic carbon assimilation in these isogenic cultivars (cf., ref. 20).

Table 1. Oxygen Inhibition of Photosynthesis by Isolated Mesophyll Protoplasts of Lollium perenne at 0.48 mM NaH14CO3 (10.5 μM CO2 at 30 C [pH 8.0])

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<th>Rate of Photosynthesis</th>
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Kinetic Properties of Protoplast Photosynthesis. O₂ inhibition of C₃ photosynthesis has been reported to be a function of the kinetic characteristics of Rbu-P₅ carboxylase-oxygenase with respect to CO₂ and O₂ (9, 14, 15). In this context, the reduced O₂ sensitivity of the tetraploid cultivar (Table I) suggests that the kinetic constants for protoplast photosynthesis in the two ryegrass lines may differ. To examine this possibility, photosynthetic ^1⁴CO₂ fixation by the 2X and 4X protoplasts was determined at various levels of NaHCO₃ and O₂ (Fig. 1). Although the rates of photosynthesis at 2% O₂ and low bicarbonate (0.25–1 mM) in the tetraploid line were always greater than in the diploid cultivar (Fig. 1 and Table I), the observed and extrapolated maximal velocities (V) were greater in the 2X protoplasts (Fig. 1, A and B). Photosynthesis in both cultivars exhibited Michaelis-Menten kinetics with respect to HCO₃⁻ at all O₂ tensions examined (Fig. 1, A and B). As expected, increasing pO₂ increased the apparent Kₗ (CO₂) for protoplast photosynthesis without altering V, indicating that O₂ was a competitive inhibitor of photosynthesis with respect to CO₂. Whereas the Kₗ (O₂) for protoplast photosynthesis was similar in both the diploid and tetraploid cultivars (392 and 404 μM, respectively), the Kₑ for HCO₃⁻ or CO₂ in the 2X line was about twice that in the 4X cultivar (Fig. 1, A and B). Thus, the kinetic properties of mesophyll protoplast photosynthesis with respect to CO₂ and O₂ (Fig. 1) are qualitatively identical to those obtained by Garrett (11) for Rbu-P₅ carboxylase-oxygenase purified from the same isogenic diploid and tetraploid lines. The physiological consequences of the observed difference in Kₑ (CO₂) for protoplast photosynthesis were further explored by examining glycolate biosynthesis and the potential for photorespiratory CO₂ efflux in the two cultivars.

Decarboxylation of Glycolate and Glycine. Mesophyll protoplasts isolated from the two isogenic lines actively decarboxylated exogenous [1-¹⁴C]glycolate and [1-¹⁴C]glycine in light and darkness (Table II). In both cultivars, ^1⁴CO₂ evolution from [1-¹⁴C]glycolate was sensitive to HBA or INH, specific inhibitors of glycolate oxidase (13, 24) and glycine decarboxylase (3, 24), respectively, whereas [1-¹⁴C]glycine decarboxylation was inhibited only by INH. These observations are consistent with the proposed sequence of carbon flow through the photorespiratory carbon oxidation cycle (5, 9). More noteworthy, the rates of glycolate and glycine decarboxylation were similar in both lines (Table II), suggesting that the two isogenic cultivars have the same potential for oxidizing glycolate and glycine to CO₂ via the photorespiratory pathway.

Glycolate Biosynthesis. As expected, preliminary experiments indicated that it was necessary to inhibit glycolate metabolism in order to quantitate the rate of glycolate formation during protoplast photosynthesis. During the standard 10-min photosynthesis assay at 21% O₂ and 0.48 mM HCO₃⁻, little glycolate accumulated (1.8–2.5 μmol [¹⁴C]glycolate/mg Chl-h) in the absence of HBA. When 1 mM HBA was included in the reaction mixture there was a marked increase in the rate of glycolate accumulation (10.2–11.7 μmol/mg Chl-h). As little as 0.8 mM HBA completely blocked the metabolic conversion of [1-¹⁴C]glycolate to ^1⁴CO₂ at 21% O₂ in both 2X and 4X protoplasts (Fig. 2). Protoplast photosynthesis at 21% O₂ and low HCO₃⁻ was also markedly inhibited by HBA (Fig. 2). At saturating levels of inhibitor, ^1⁴CO₂ fixation was reduced by about 40% in the diploid protoplasts compared to 30% in the tetraploid. Concomitant with this decrease in photosynthesis under conditions favoring glycolate formation (i.e. low pCO₂ and high pO₂), there was an increase in the inhibition of photosynthesis by 21% O₂ in the presence of 1 mM HBA (from 36 to 60% and 28 to 48% in the 2X and 4X lines, respectively) (data not shown). In
contrast, under assay conditions in which glycolate biosynthesis would largely be prevented (2% O₂ or 10 mM bicarbonate), HBA had little effect on either 2X or 4X protoplast 14CO₂ fixation (less than 4% inhibition; data not shown) indicating that the compound does not inhibit photosynthesis per se. Essentially identical HBA (and INH) effects on photosynthesis have previously been reported with an isolated soybean leaf cell system (24).

The decrease in the rate of 14CO₂ fixation at low pCO₂ and 21% O₂ in the presence of HBA (Fig. 2 and ref. 24) is likely due to the fact that for every four carbon atoms entering the photosynthetic carbon oxidation cycle as glycolate, at least one is released as CO₂ with the remaining carbon recycling back into the Calvin cycle (2, 5). When glycolate oxidation is inhibited by HBA under conditions which promote glycolate biosynthesis and thus photorespiration, carbon diverted from the Calvin cycle into the photosynthetic pathway accumulates as glycolate with little recycling back into the C₂ cycle, thus presumably leading to a depletion of Calvin cycle intermediates, particularly Rbu-P₂, and a concomitant inhibition of photosynthesis (24). The observation that the inhibitory effects of HBA on ryegrass protoplast photosynthesis at 21% O₂ and 0.48 mM HCO₃⁻ are essentially negated by the addition of 0.5 mM ribose-5-P, a C₃ cycle precursor of Rbu-P₂, to the reaction medium supports this contention (data not shown).

Thus, inhibition of glycolate metabolism under photosynthetic conditions is severely deleterious to photosynthesis and it is essential that glycolate, once synthesized, be metabolized if normal photosynthesis rates are to be maintained (24). It is tempting to speculate that the rate of glycolate formation relative to photosynthesis at 21% O₂ and low pCO₂ in the 2X cultivar is greater than in the 4X line, resulting in a greater depletion of Calvin cycle intermediates in the presence of HBA and thus an increased HBA inhibition of photosynthesis as observed in the diploid protoplasts (Fig. 2).

Direct evidence for this supposition was obtained from comparative studies of the rates of 14CO₂ fixation and [14C]glycolate accumulation by diploid and tetraploid protoplasts as a function of H₁₄CO₃⁻ concentration at 21% O₂ and saturating levels of HBA (1 mM; Fig. 2). Under these conditions photosynthesis increased with increasing bicarbonate concentration up to 5 mM (Fig. 3A).

In contrast, the rate of [14C]glycolate formation reached a maximum at 0.5 and 0.25 mM HCO₃⁻ in the 2X and 4X protoplasts, respectively, and then decreased in both lines with increasing bicarbonate concentration (Fig. 3A). The rate of [14C]glycolate accumulation at 5 mM HCO₃⁻ and 21% O₂ approached the control rate at 2% O₂ and 0.48 mM HCO₃⁻ (about 0.8 μmol/mg Chl-h). The reason(s) for the observed difference between the two cultivars with respect to the bicarbonate concentration required for maximal glycolate synthesis at 21% O₂ (Fig. 3A) is not known, although it is likely a function of the concentration of HCO₃⁻ required to build up the stromal pools of Calvin cycle intermediates from which glycolate is derived. This suggestion is consistent with the observed difference in the Kₘ (CO₂) for protoplast photosynthesis between the two cultivars (Fig. 1) in that the 4X line, because of its reduced Kₘ (CO₂), would presumably require a lower HCO₃⁻ concentration to saturate its C₃ cycle carbon pools compared to that in the 2X protoplasts. The observation that the absolute rate of [14C]glycolate synthesis at very low levels of bicarbonate (0.1 mM) was significantly higher in the tetraploid (43%) than in the diploid line (Fig. 3A) further supports this contention.

**Fig. 2.** Effect of HBA concentration on rates of photosynthesis at 21% O₂ and 0.48 mM H₁₄CO₃⁻ (A, △) and [14C]glycolate decarboxylation at 21% O₂ ( ●, ○) by illuminated mesophyll protoplasts isolated from isogenic diploid (△, ○) and tetraploid (●, △) cultivars of ryegrass. Decarboxylation assays included 50 mM D,L-glyceraldehyde and 10 mM maleate to prevent endogenous refixation of released 14CO₂ by Rhu-P₂ carboxylase and PEP carboxylase, respectively (18, 20, 26). The control rates were 140 and 185 μmol 14CO₂ fixed/mg Chl-h, and 17.3 and 16.5 μmol 14CO₂ evolved/mg Chl-h for diploid and tetraploid lines, respectively.

**Fig. 3.** Effect of H₁₄CO₃⁻ concentration (0.1-5.0 mM) on simultaneous rates of photosynthesis (●, ○) and [14C]glycolate formation (●, □) (A), and ratio of total [14C]glycolate synthesized to total 14CO₂ fixed (B) by diploid (●, □) and tetraploid (○, △) ryegrass mesophyll protoplasts in presence of 21% O₂ and 1 mM HBA. Inset shows percentage of total water-soluble 14C fixed entering [14C]glycolate pool in presence of 1 mM HBA.
The similar maximal rates of $[^{14}]C$glycolate formation in the two cultivars at 21% O$_2$ (Fig. 3A) is intriguing in view of the observed differences in $K_m$ (CO$_2$) and O$_2$ inhibition of protoplast photosynthesis (Fig. 1 and Table I). We have examined other parameters in this protoplast system that reflect the magnitude of photorespiratory glycolate formation relative to photosynthesis in the two cultivars. Inasmuch as the rate of $^{14}$CO$_2$ fixation by the protoplasts increased with increasing concentration of HCO$_3^-$ whereas glycolate synthesis did not increase above 0.48 mm HCO$_3^-$ (Fig. 3A), the ratio of total $[^{14}]$Cglycolate synthesized to total $^{14}$CO$_2$ fixed during the same 10-min interval decreased sharply with increasing bicarbonate (Fig. 3B). More noteworthy, the ratio of the rate of $[^{14}]$Cglycolate formation to $^{14}$CO$_2$ fixation under active photorespiratory conditions (0.1–1.0 mm HCO$_3^-$, 21% O$_2$) was not similar in the two cultivars, but was about two times greater in the diploid line compared to the tetraploid (Fig. 3B). A similar difference between the 2x and 4x cultivars was also observed with respect to the per cent of the total fixed $^{14}$C entering the glycolate pool in the presence of HBA (Fig. 3B, inset). From these data it is likely that a significant difference exists between the two isogenic cultivars with respect to the rate of photorespiratory glycolate formation relative to photosynthesis, presumably reflecting the presence of Rbu-P$_2$ carboxylase-oxygenase with dissimilar kinetic properties (11).

In the experiments summarized in Figure 3 the specific radioactivity of the photosynthesized $[^{14}]$Cglycolate was assumed to be identical to that of the added H$^{14}$CO$_3^-$ on a carbon basis. To obtain a more accurate estimate of $[^{14}]$Cglycolate formation the protoplasts were fed a C$_3$ cycle precursor of glycolate, [U-$^{14}$C]ribose-5-P, in the absence of HCO$_3^-$/CO$_2$ and subsequently analyzed for $[^{14}]$Cglycolate accumulation based on the known specific radioactivity of the exogenous [U-$^{14}$C]ribose-5-P (6). In preliminary experiments, no significant accumulation of labeled glycolate was observed from [U-$^{14}$C]ribose-5-P unless HBA was included in the protoplast reaction mixture, similar to the situation in the H$^{14}$CO$_3^-$ experiments. In the presence of 1 mm HBA, the rates of $[^{14}]$Cglycolate accumulation from 0.5 mm [U-$^{14}$C]ribose-5-P were similar between the two lines of protoplasts, being 0.89 and 0.81 $\mu$mol/mg Chl-h at 0% O$_2$, 7.9 and 8.0 at 21% O$_2$, and 17.4 and 17.1 at 49% O$_2$ in the 2x and 4x cultivars, respectively. These data independently confirm the results obtained from the H$^{14}$CO$_3^-$ experiments (Fig. 3A) in that the maximal rates of protoplast glycolate synthesis in the presence of HBA were similar in the isogenic diploid and tetraploid cultivars.

Although the general protocol employed in measuring $[^{14}]$Cglycolate accumulation during photosynthesis by the ryegrass protoplasts has been used in similar studies with other isolated leaf cell types (6, 24), the assay system is somewhat artificial in that the synthesis of glycolate must be followed in the presence of a glycolate oxidase inhibitor to prevent the subsequent metabolism of glycolate in the peroxisomes. Since there is unequivocal evidence that glycolate biosynthesis occurs in the chloroplast (12) and that the plastid has a limited capacity for metabolizing glycolate (8, 12), this metabolite may be regarded as an end product of chloroplast photosynthesis. The isolated intact chloroplast provides a convenient and relatively simple system for quantitating glycolate formation during photosynthesis without the need for a glycolate oxidase inhibitor (8). Direct measurements of $[^{14}]$Cglycolate formation during $^{14}$CO$_2$ fixation by isolated ryegrass chloroplasts at 0.48 mm H$^{14}$CO$_3^-$ and 21% O$_2$ indicated that the rate of glycolate synthesis in the 2x line was about 45% greater than in the 4x cultivar (Table III). As in the protoplast studies (Fig. 3B), both the percentage of the total fixed carbon entering the glycolate pool and the ratio of the rate of $[^{14}]$Cglycolate synthesis to $^{14}$CO$_2$ fixation during the same 10-min interval were about two times greater in the plastids isolated from the diploid line compared to those from the tetraploid (Table III).

### CONCLUSIONS

The results from the isolated protoplast and intact chloroplast studies indicate that a significant difference exists between the isogenic diploid and tetraploid cultivars of ryegrass with respect to the rate of photorespiratory glycolate formation relative to photosynthesis (Fig. 3 and Table III), presumably reflecting the presence of Rbu-P$_2$ carboxylase-oxygenase with dissimilar kinetic properties (ref. 11 and Fig. 1). In contrast, it seems that ploidy level has had little effect on the metabolic capacity for oxidizing glycolate and glycine to CO$_2$ via the photorespiratory carbon oxidation cycle (Table II). Although increases in ploidy have been shown to alter quantitatively a number of photosynthetic related properties in plants (17, 25), including Rbu-P$_2$ carboxylase activity (17), the 4x ryegrass cultivar appears to be an exception in that it differs qualitatively from its isogenic diploid progenitor at least with respect to the kinetic properties of Rbu-P$_2$ carboxylase-oxygenase. It remains to be established how a doubling of ploidy brought about such a qualitative change in this bifunctional enzyme.

Andrews and Lorimer (2) have suggested that oxygenation of Rbu-P$_2$ and thus photospiration are the unavoidable consequence of the active site chemistry of Rbu-P$_2$ carboxylase-oxygenase and that the two competing activities are unlikely to be differentially regulated. However, temperature (4, 14, 15) and Mg$^{2+}$/Mn$^{2+}$ (27; Rejda and Chollet, unpublished data; Jordan and Ogren, personal communication) have been shown to affect differentially the two activities in vitro. Large differences in the size, shape, and charge distribution in CO$_2$ and O$_2$ provide some optimism that the binding of the two gaseous substrates can be differentially affected (15). Since the partial charges of the carbon and oxygen atoms in CO$_2$ are +0.22 and -0.11, respectively, whereas the atoms of molecular oxygen have no partial charge, Ogren (15) has suggested that ionic alterations at the active site of Rbu-P$_2$ carboxylase are more likely to influence CO$_2$ binding than that of O$_2$, a speculation offering one possible explanation for the altered $K_m$ (CO$_2$) in the ryegrass cultivars (11). Further studies with crystalline and noncrystalline Rbu-P$_2$ carboxylase-oxygenases isolated from the two isogenic cultivars are in progress in our laboratory to determine the molecular basis of this kinetic difference.

### LITERATURE CITED

5. BERRY, J.A. AND OSMOND, C.B. 1978. Fixation of $^{14}$O$_2$ during photospira-
27. Wildner GF, J Henkel 1979 The effect of divalent metal ions on the activity of Mg²⁺ depleted ribulose-1,5-bisphosphate carboxylase. Planta 146: 223–228