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

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ABSTRACT
Photosynthetic 14CO2 fixation, 14C[glycolate formation, and the decarboxylation of \( \text{[1-14C]} \)glycolate and \( \text{[1-14C]} \)glycine by leaf mesophyll protoplasts isolated from isogenic diploid and tetraploid cultivars of ryegrass (Lolium perenne L.) were examined. Treatment of the CO2 uptake 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 \( K_m \) (CO2) for photosynthesis by the diploid protoplasts was about twice that of the tetraploid line. In contrast, the \( K_i \) (O2) for protoplast photosynthesis was similar in both cultivars, as was the potential for oxidizing glycolate and glycine to CO2 via the photorespiratory carbon oxidation cycle. Although the maximal rates of glycolate accumulation by the isolated protoplasts in the presence of 21% O2 and a glycolate oxidase inhibitor were similar in the two cultivars, the percentage of total fixed 14CO2 entering the \( \text{[1-14C]} \)glycolate pool and the ratio of the rate of \( \text{[1-14C]} \)glycolate formation to \( \text{[14C]} \)CO2 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 \( K_m \) ( CO2) of purified ribulose bisphosphate carboxylase-oxygenase by about one-half without affecting the \( K_i \) (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 \( K_m \) (CO2) of Rbu-P2, a carboxylase-oxygenase (EC 4.1.1.39) purified from various cultivars of ryegrass, including an isogenic diploid and tetraploid line. Whereas the \( K_m \) (CO2) (carboxylase) and \( K_i \) (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, 21 C day/16 C 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 (10), suspended in 0.3 mM 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 mM 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. \( ^{14} \text{C} \)CO2 fixation, \( \text{[1-14C]} \)glycolate and \( \text{[1-14C]} \)glycine decarboxylation and \( \text{[1-14C]} \)glycolate synthesis assays were performed in serum-stoppered glass vials containing a standard CO2-free reaction mixture of 0.3 mM sorbitol, 50 mM Tricine, 1 mM MgCl2, 1 mM MnCl2, 2 mM KH2PO4 (0.1 mM 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
(400-w Ceramalux, Westinghouse), giving a quantum flux density of about 5-10.5 x 10^2 μE m^-2 s^-1 (400-700 nm) at the surface of the reaction vials.

**Photosynthetic 14CO2 Fixation.** The reaction vials contained the standard assay medium, leaf slices, or isolated protoplasts or chloroplasts (3-9 μg Chl), and 0.25-4.0 mm NaH14CO3 (2.5 Ci/mol) (New England Nuclear) in a final volume of 0.5 ml. Where indicated, various enzyme inhibitors (adjusted to pH 8.0 with KOH) were included in the standard assay medium. The sealed reaction vials containing the assay medium and leaf slices, protoplasts or chloroplasts were flushed at 25 C for 15-20 min with humidified O2/N2 mixtures (Matheson Gas Products), preilluminated for 5 min, and the assays initiated by injecting NaH14CO3. The reactions were terminated after 10 min by injecting 0.25 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).

**Glycolate and Glycerine Decarboxylation.** Decarboxylation of glycolate 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]glycolate (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 absence or presence 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 14C-substrate. All values were corrected for nonenzymic decarboxylation with boiled protoplast controls.

**Glycolate Synthesis.** Photosynthetic [14C]glycolate formation by isolated protoplasts and chloroplasts was followed using NaH14CO3 or [U-14C]ribos-5-P (Schwarz/Mann). [U-14C]Ribo-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]glycolate formation were identical to those described above for 14CO2 fixation except that the protoplasm assay medium routinely contained 1 mm HBA (adjusted to pH 8.0 with KOH) to inhibit completely the subsequent metabolism of glycolate (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]ribose-5-P (final concentration, 0.5 mm; 0.3 Ci/ml) 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 unfixed 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 μl of carrier glycolate were 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 20,000 g 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 glycolate-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 redissolved in H2O followed by filtration through a 0.22-μm pore-sized filter. The filtrate was chromatographed on precoated TLC plates of Cellulose 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 N NaHCO3 to prevent volatilization of glycolic acid, air-dried, and scanned for radioactivity. The radioactive peak which co-chromatographed with authentic glycolate was eluted and dpm determined by liquid scintillation spectroscopy (6, 21). Recovery of [1-14C]glycolate 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 glycolate 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 importantly, glycolate is well resolved (RF = 0.49) from glycerate (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 glycolate oxidase (13, 24), and INH for glyceric 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 RuB-P2 carboxylase on a Chl basis was similar in both lines (592 to 618 μmol/mg Chl-h). The presence of a C4-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 1 shows the effect of pO2 on photosynthesis by isolated leaf mesophyll protoplasts at low HCO3- (0.48 mm, 10.5 μmol 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 2, 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 1) 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 Loliun perenne at 0.48 mm NaH14CO3 (10.5 μmol CO2 at 30 C [pH 8.0])**

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Rate of Photosynthesis</th>
<th>Oxygen Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>O2</td>
<td>O2</td>
</tr>
<tr>
<td></td>
<td>2%</td>
<td>21%</td>
</tr>
<tr>
<td>Diploid</td>
<td>164</td>
<td>105</td>
</tr>
<tr>
<td>Control</td>
<td>160</td>
<td>102</td>
</tr>
<tr>
<td>+Maleate, 10 mm</td>
<td>210</td>
<td>154</td>
</tr>
<tr>
<td>+Maleate, 10 mm</td>
<td>212</td>
<td>156</td>
</tr>
</tbody>
</table>

Results represent mean of four independent experiments. Essentially identical results were obtained in similar photosynthesis experiments with thin leaf slices.
Kinetic Properties of Protoplast Photosynthesis. O₂ inhibition of C₃ photosynthesis has been reported to be a function of the kinetic characteristics of Rbg-P₇ 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 [¹⁴C]CO₂ fixation by the 2× and 4× protoplasts was determined at various levels of NaH[¹⁴C]CO₃ 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 (Vₘ) were greater in the 2× 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 Kₘ (CO₂) for protoplast photosynthesis without altering Vₘ, indicating that O₂ was a competitive inhibitor of photosynthesis with respect to CO₂. Whereas the Kₘ (O₂) for protoplast photosynthesis was similar in both the diploid and tetraploid cultivars (392 and 404 μm, respectively), the Kₘ for HCO₃⁻ or CO₂ in the 2× line was about twice that in the 4× 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 Rbg-P₇ carboxylase-oxygenase purified from the same isogenic diploid and tetraploid lines. The physiological consequences of the observed difference in Kₘ (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 [¹⁴C]glycolate and [¹⁴C]glycine in light and darkness (Table II). In both cultivars, [¹⁴C]CO₂ evolution from [¹⁴C]glycolate was sensitive to HBA or INH, specific inhibitors of glycolate oxidase (13, 24) and glycine decarboxylase (3, 24), respectively, whereas [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 [¹⁴C]glycolate to [¹⁴C]CO₂ at 21% O₂ in both 2× and 4× protoplasts (Fig. 2). Protoplast photosynthesis at 21% O₂ and low HCO₃⁻ was also markedly inhibited by HBA (Fig. 2). At saturating levels of inhibitor, [¹⁴C]CO₂ 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 2× and 4× lines, respectively) (data not shown). In

Table II. Decarboxylation of [¹⁴C]Glycolate and [¹⁴C]Glycine by Isolated Mesophyll Protoplasts of Lolium perenne at 21% O₂

<table>
<thead>
<tr>
<th>Assay Conditions</th>
<th>Diploid</th>
<th>Tetraploid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>Glycolate, 5 mm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ HBA, 2 mm</td>
<td>17.4</td>
<td>15.9</td>
</tr>
<tr>
<td>+ INH, 5 mm</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>Glycine, 5 mm</td>
<td>19.4</td>
<td>19.9</td>
</tr>
<tr>
<td>+ HBA, 2 mm</td>
<td>18.7</td>
<td>17.6</td>
</tr>
<tr>
<td>+ INH, 5 mm</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results represent mean of two independent experiments, corrected for boiled protoplast controls.

![Fig. 1. Double reciprocal plots of diploid (A) and tetraploid (B) ryegrass mesophyll protoplast photosynthesis versus H¹⁴CO₃⁻ concentration (0.25–4.0 mM) at 2 (V), 21 (D), 49 (C), and 100% (Δ) O₂. Slopes, y-intercepts, and best fit lines were determined by linear regression analysis. Kₘ (CO₂) and Kₘ (O₂) values were estimated from Lineweaver-Burk and Dixon plots, respectively. Results are representative of three independent experiments for which mean Kₘ (CO₂) values were 37.4 (±3.5) and 19.5 μM (±0.8), and mean Kₘ (O₂) values were 37.1 (±4.4) and 380 μM (±45) for 2× and 4× protoplasts, respectively.](https://www.plantphysiol.org/doi/10.1104/pp.65.3.491)
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 re-
ported 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 photorespiratory
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 photorespiratory pathway accumulates as glycolate with little recy-
cling back into the C₃ cycle, thus presumably leading to a depletion
of Calvin cycle intermediates, particularly Ru-bP₂, and a concom-
itant 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 Ru-bP₂, to
the reaction medium supports this contention (data not shown).

Thus, inhibition of glycolate metabolism under photorespiratory
conditions is severely deleterious to photosynthesis and it is essen-
tial 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 photosyn-
thesis 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 com-
parative 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 (Fig. 3A). 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]gly-
colate 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 concentation of HCO₃⁻
required to build up the stromal pools of Calvin cycle intermedi-
ates 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 conten-
tion.
The similar maximal rates of [14C]glycolate formation in the two cultivars at 21% O₂ (Fig. 3A) is intriguing in view of the observed differences in Km (CO₂) and O₂ inhibition of protoplast photosynthesis (Fig. 1 and Table I). We have examined other parameters in this protoplast system that reflect the magnitude of photosynthetic glycolate formation relative to photosynthesis in the two cultivars. Inasmuch as the rate of 14CO₂ fixation by the protoplasts increased with increasing concentration of HCO₃⁻ whereas glycolate synthesis did not increase above 0.48 mm HCO₃⁻ (Fig. 3A), the ratio of total [14C]glycolate synthesized to total 14CO₂ fixed during the same 10-minute interval decreased sharply with increasing bicarbonate (Fig. 3B). More noteworthy, the ratio of the rate of [14C]glycolate formation to 14CO₂ fixation under active photorespiratory conditions (0.1–1.0 mm HCO₃⁻, 21% O₂) was not similar in the two cultivars, but was about twice 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 14C 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 diploid cultivars with respect to the rate of photosynthetic glycolate formation relative to photosynthesis, presumably reflecting the presence of Rbu-P₂ carboxylase-oxygenase with dissimilar kinetic properties (11).

In the experiments summarized in Figure 3 the specific radioactivity of the photosynthesized [14C]glycolate was assumed to be identical to that of the added HCO₃⁻ on a carbon basis. To obtain a more accurate estimate of [14C]glycolate formation the protoplasts were fed a C₅ cycle precursor of glycolate, [U-14C]-ribulose-5-P, in the absence of HCO₃⁻/CO₂ and subsequently analyzed for [14C]glycolate accumulation based on the known specific radioactivity of the exogenous [U-14C]-ribulose-5-P (6). In preliminary experiments, no significant accumulation of labeled glycolate was observed from [U-14C]ribulose-5-P unless HBA was included in the protoplast reaction mixture, similar to the situation in the HCO₃⁻/CO₂ experiments. In the presence of 1 mm HBA, the rates of [14C]glycolate accumulation from 0.5 mm [U-14C]ribulose-5-P were similar between the two lines of protoplasts, being 0.89 and 0.81 μmol/mg Chl-h at 2% O₂, 7.9 and 8.0 at 21% O₂, and 17.4 and 17.1 at 49% O₂ in the 2x and 4x cultivars, respectively. These data independently confirmed the results obtained from the HCO₃⁻/CO₂ 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 [14C]glycolate 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 [14C]glycolate formation during 14CO₂ fixation by isolated ryegrass chloroplasts at 0.48 mm HCO₃⁻ and 21% O₂ 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 [14C]glycolate synthesis to 14CO₂ fixation during the same 10-minute interval were about two times greater in the plasts 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 photosynthetic glycolate formation relative to photosynthesis (Fig. 3 and Table III), presumably reflecting the presence of Rbu-P₂ 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 CO₂ via the photosynthetic oxygenic 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-P₂ 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-P₂ 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-P₂ and thus photospiration are the unavoidable consequence of the active site chemistry of Rbu-P₂ carboxylase-oxygenase and that the two competing activities are unlikely to be differentially regulated. However, temperature (4, 14, 15) and Mg⁺⁺/Mn⁺⁺ (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 CO₂ and O₂ 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 CO₂ 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-P₂ carboxylase are more likely to influence CO₂ binding than that of O₂, a speculation offering one possible explanation for the altered Km (CO₂) in the ryegrass cultivars (11). Further studies with crystalline and noncrystalline Rbu-P₂ 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 JA, CB Osmond, GH Lorimer 1978 Fixation of 14O₂ during photosyn-

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**Table III. Photosynthesis and Glycolate Formation by Isolated Chloroplasts of Lolium perenne at 0.45 mm NaH14CO₃ (10.5 μm CO₂ at 30°C (pH 8.0)) and 21% O₂.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diploid</th>
<th>Tetraploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>[14C]CO₂ Fixation (μmol/mg Chl-h)</td>
<td>50.7</td>
<td>69.0</td>
</tr>
<tr>
<td>[14C]Glycolate Formation (μmol/mg Chl-h)</td>
<td>9.1</td>
<td>6.2</td>
</tr>
<tr>
<td>[14C]Glycolate (% of total water-soluble)</td>
<td>37.0</td>
<td>18.4</td>
</tr>
<tr>
<td>Rate of glycolate formation/Rate of [14C]CO₂ fixation × 100</td>
<td>17.9</td>
<td>9.0</td>
</tr>
</tbody>
</table>
27. Wildner GF, J Hensek 1979 The effect of divalent metal ions on the activity of Mg2+ depleted ribulose-1,5-bisphosphate oxygenase. Planta 146: 223–228