THE EFFECT OF OXYGEN ON $^{14}CO_2$ FIXATION IN MESOPHYLL CELLS ISOLATED FROM DIGITARIA SANGUINALIS (L.) SCOP. LEAVES*

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SUMMARY

Mesophyll cells were isolated from fully-expanded leaves of Digitaria sanguinalis (L.) Scop. by a combined maceration-filtration technique. In the presence of pyruvate, photosynthetic $^{14}CO_2$ uptake in the isolated cells was not inhibited by atmospheric levels of oxygen. In contrast, superatmospheric levels of oxygen substantially inhibited the light-dependent fixation of $^{14}CO_2$. These oxygen effects are similar to those observed with intact C_4 leaves and suggest that the lack of inhibition of C_4 photosynthesis by atmospheric levels of oxygen results from the relative oxygen-insensitivity of the phosphopyruvate carboxylase-CO_2 pump in the mesophyll.

INTRODUCTION

Higher plants can be divided into two major groups, C_3 and C_4 species, based on several anatomical, physiological, and biochemical criteria, including the oxygen sensitivity of photosynthesis. In C_3 plants such as soybean, CO_2 fixation in air is inhibited by about 30% compared to the rate in 1% oxygen[1]. In contrast, photosynthesis in C_4 species such as crabgrass and maize is substantially inhibited by only superatmospheric levels of oxygen[2,3]. In the first group of plants, CO_2 is initially fixed into 3-PGA by RuDP carboxylase (EC 4.1.1.39). In crabgrass and maize, atmospheric CO_2 is initially fixed in the mesophyll cells by PEP carboxylase

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Abbreviations: Chl, chlorophyll; DTT, dithiothreitol; PEP, phosphoenolpyruvate; 3-PGA, 3-phosphoglycerate; RuDP, ribulose-1,5-diphosphate; Tricine, N-tris-(hydroxymethyl)methylglycine.

(EC4.1.1.31). The resultant oxaloacetate is predominantly reduced to malate, and the malate is transported into the bundle sheath cell layer where it is decarboxylated to CO_2 and pyruvate by "malic" enzyme (EC 1.1.1.40). The pyruvate is transported back to the mesophyll to regenerate PEP by the pyruvate, P_i dikinase reaction, thus completing the C_4 cycle, and the CO_2 is refixed by RuDP carboxylase into 3-PGA, which flows through the Calvin cycle to carbohydrate[4-8]. The PEP carboxylase-"malic" enzyme system presumably functions as a CO_2 pump, increasing the CO_2 concentration in the bundle sheath above that in free equilibrium with the leaf[9].

It has been previously reported that photosynthetic CO_2 fixation in isolated maize bundle sheath cells is substantially inhibited by atmospheric levels of oxygen, and this reversible inhibition is relieved by increased levels of CO_2[10]. It was suggested that the inhibitory effect of oxygen was on RuDP carboxylase[10], since this enzyme is localized in this cell type[4-8,10] and is competitively inhibited by oxygen with respect to CO_2[11].

The experiments reported here demonstrate that light-induced $^{14}CO_2$ fixation in isolated crabgrass mesophyll cells is not inhibited by atmospheric levels of oxygen. These observations strongly support the previous proposals[10,11] that the insensitivity of photosynthesis to atmospheric levels of oxygen in the leaves of C_4 plants results from the unhindered operation of the CO_2 pump in the mesophyll which serves to concentrate CO_2 in the bundle sheath, the site of RuDP carboxylase.

MATERIALS AND METHODS

Mesophyll cells were isolated at 4°, following the procedure of Edwards and Black[12], from fully-expanded leaves of 3- to 4-week-old Digitaria sanguinalis (L.) Scop. (hairy crabgrass) grown at 2400 ft-c. The isolated cells were suspended and assayed in 0.33 M d-sorbitol, 50 mM Tricine, 3 mM $MgCl_2$, 2 mM

TABLE I. Enzyme activities in mesophyll cell extracts.

Enzyme	μmoles $^{14}CO_2$ fixed/mg Chl/hr
RuDP carboxylase	3
PEP carboxylase	331

TABLE II. Effect of exogenous substrates on mesophyll cell $^{14}CO_2$ fixation.

Exogenous Substrate	Light	Dark
	μmoles $^{14}CO_2$ fixed/mg Chl/hr	
No addition	0.00	0.00
5 mM ribose-5-P	0.14	0.07
5 mM pyruvate	8.85	0.00
5 mM PEP	554	502

Fixation was assayed for 4 min at 5 mM $NaH^{14}CO_3$ (0.4 μCi/μmole), ±1400 ft-c in air; rates corrected for zero-time controls.

$NaNO_3$, 1 mM Na_2EDTA, 1 mM $MnCl_2$, 1 mM K_2HPO_4 (final pH of the medium adjusted to 8.0 with NaOH), and 5 mM freshly-prepared DTT. All assays were performed within 90 min after isolation.

Photosynthetic CO_2 fixation by the isolated cells was determined by $^{14}CO_2$ incorporation at 25° and 1400 ft-c. Unless noted otherwise, the reaction vessels contained assay medium, mesophyll cells (4-9 μg Chl), 5 mM pyruvate or PEP, and 1 mM $NaH^{14}CO_3$ (2-4 μCi/μmole) in a final volume of 1.0 ml. Vessels containing cells were preilluminated and gassed with vigorous shaking for 6 min and sealed. The reactions were initiated by injecting $NaH^{14}CO_3$ and terminated after 4 min by injecting 0.1 ml of 0.13 M 2,4-dinitrophenylhydrazine in 6 N HCl. Contents of the vessels were thoroughly mixed, aliquots were dried under N_2 at 25°, and dpm determined by liquid scintillation

TABLE III. Effect of oxygen on mesophyll cell $^{14}CO_2$ fixation.

Substrate	Gas phase	Rate[a]	Per cent Inhibition
5 mM pyruvate	2% O_2	5.42	--
	20% O_2	6.00	0
	100% O_2	1.88	65
5 mM PEP	2% O_2	285	--
	20% O_2	304	0
	100% O_2	297	0

[a] μmoles $^{14}CO_2$ fixed/mg Chl/hr.

spectroscopy. Commercially-prepared and analyzed oxygen-helium mixtures were used in the various oxygen experiments.

For enzyme assays, the isolated cells were frozen in liquid nitrogen and homogenized and filtered as described by Chen and coworkers[5]. The activity of RuDP carboxylase was assayed as previously described[13]. PEP carboxylase was assayed in a 1.0 ml reaction medium containing 40 mM tris-HCl (pH 8.0), 5 mM $MgCl_2$, 5 mM sodium glutamate, 5 mM $NaH^{14}CO_3$ (0.3 μCi/μmole), 2.5 mM DTT, 2 mM PEP, 0.1 mM Na_2 EDTA, 10 units of glutamate-oxaloacetate transaminase (EC 2.6.1.1; purchased from Worthington Biochemical Corp.), and 0.1 ml of the crude filtrate. The reactions were initiated with PEP, run for 3 min at 30°, and stopped with 0.1 ml of 6 N acetic acid. The samples were dried and counted as described elsewhere[13].

For the determination of chlorophyll, aliquots of the cells were dried under a stream of N_2 at 25° in the dark. The residues were taken up in 96% aqueous ethanol, sonicated

for 10 min, centrifuged, and the clarified ethanol extract used for chlorophyll determinations[14].

RESULTS

The contamination of mesophyll cell preparations by bundle sheath cells was judged to be negligible, as determined by light microscopy and the relative activities of mesophyll and bundle sheath marker enzymes. Microscopically, the rounded or egg-shaped mesophyll cells were readily distinguished from the rectangular bundle sheath cells on the basis of shape and the scattered distribution of chloroplasts in the former as compared to the densely-packed plastid distribution in the latter[4,12]. The near lack of RuDP carboxylase activity in mesophyll cell extracts, as compared to the activity of PEP carboxylase (Table I), was also indicative of a high degree of purity[4-8,10].

Isolated crabgrass mesophyll cells lacked the capacity for endogenous photosynthetic $^{14}CO_2$ fixation (Table II). Exogenous ribose-5-P had little effect on the rate of $^{14}CO_2$ uptake in the isolated cells, whereas pyruvate and PEP greatly stimulated the fixation of $^{14}CO_2$ (Table II). The lack of a substantial stimulation of mesophyll cell $^{14}CO_2$ uptake by ribose-5-P, a Calvin cycle intermediate, provides further evidence for the absence of bundle sheath contamination of the preparations[4,6,15].

The effect of oxygen on $^{14}CO_2$ fixation in isolated crabgrass mesophyll cells is given in Table III. Pyruvate-dependent $^{14}CO_2$ uptake was not inhibited by atmospheric levels of oxygen. Under pure oxygen, $^{14}CO_2$ fixation was inhibited by about 65% and the inhibition was not reduced by increasing the bicarbonate concentration to 5 mM (data not shown). In the presence of PEP, $^{14}CO_2$ uptake in the cells was not inhibited by oxygen tensions up to 100% (Table III).

DISCUSSION

Mesophyll cells isolated from fully-expanded leaves of

crabgrass lack the capacity for endogenous photosynthetic $^{14}CO_2$ uptake[4] (Table II). This inability is consistent with the compartmentation scheme for C_4 photosynthesis advanced by Hatch and coworkers[8] and by others[4-7,15] in which the complete C_4 cycle is compartmented between the mesophyll and bundle sheath cells. According to this scheme, the carboxylation of PEP in isolated mesophyll cells would not be cyclic due to the absence of "malic" enzyme[4,5,7,8] which provides the pyruvate for the regeneration of PEP, and thus $^{14}CO_2$ fixation would require priming with exogenous substrate.

The lack of an inhibitory effect of atmospheric levels of oxygen on $^{14}CO_2$ uptake in isolated crabgrass mesophyll cells (Table III) is similar to the findings with intact leaf tissue from C_4 species[2,3,16]. In contrast, photosynthesis in isolated maize bundle sheath cells is substantially inhibited by 21% oxygen[10], probably by a competitive inhibition of RuDP carboxylase with respect to CO_2[10,11]. It is proposed that the lack of inhibition of photosynthesis in intact C_4 leaves by atmospheric levels of oxygen results from the relative oxygen-insensitivity of the PEP carboxylase-CO_2 pump in the mesophyll. This CO_2 concentrating mechanism[9] serves to increase the CO_2/O_2 ratio in the bundle sheath, thus allowing CO_2 to more effectively compete with oxygen for RuDP carboxylase.

At levels of oxygen greatly exceeding atmospheric, pyruvate-dependent $^{14}CO_2$ fixation in isolated mesophyll cells is substantially inhibited (Table III), and this inhibitory effect of oxygen is not reduced by increasing the level of CO_2. These observations are also similar to the findings with intact C_4 leaves[2,3]. Although the mechanism of this inhibitory effect of oxygen on $^{14}CO_2$ uptake is unknown, it is probably not due to a direct inhibition of PEP carboxylase since: (a) <u>in vitro</u> PEP carboxylase activity is unaffected by oxygen[11], and (b) PEP-dependent $^{14}CO_2$ uptake in isolated crabgrass mesophyll

cells is not inhibited by 100% oxygen (Table III). One possible site of oxygen inhibition in the mesophyll could be the pyruvate, P_i dikinase reaction which converts pyruvate to PEP. The *in vitro* activity of this sulfhydryl-sensitive enzyme has been reported to be inhibited by oxygen[17].

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