THE EFFECT OF OXYGEN ON CO_2 FIXATION BY MESOPHYLL PROTOPLAST EXTRACTS OF C_3 AND C_4 PLANTS.*

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SUMMARY: Oxygen inhibits CO_2 fixation by mesophyll protoplast extracts of the C_3 plants, Hordeum vulgare and Triticum aestivum, but stimulates the pyruvate induced CO_2 fixation by mesophyll protoplast extracts of the C_4 plants Digitaria sanguinalis and Urochloa panicoides. The former is reversed by increased levels of bicarbonate, whereas the latter effect is independent of bicarbonate concentration. The results are consistent with the proposal that oxygen inhibits C_3 photosynthesis by competing with CO_2 in the RuDP carboxylase/oxygenase system. The oxygen enhancement of C_4 mesophyll photosynthesis is proposed to be due to pseudocyclic electron flow supplying additional ATP for the CO_2 fixation process.

C_3 and C_4 plants** can be distinguished on the basis of several anatomical and biochemical criteria (1). In C_3 plants, atmospheric CO_2 is fixed directly in the mesophyll cells by RuDP carboxylase whereas C_4 plants have adapted a complex mechanism to concentrate CO_2 in the bundle sheath cells - the site of net CO_2 fixation by RuDP carboxylase. In C_4 plants, atmospheric CO_2 is fixed initially in the mesophyll cells by PEP carboxylase into C_4 acids which are then transported to the bundle sheath cells where they are decarboxylated, forming a concentrated CO_2 pool for net refixation in the Calvin pathway (1). The 3-carbon product of the decarboxylation reaction would then be transported back to the mesophyll cells to keep the C_4 pathway operating. This CO_2 concentrating mechanism has been proposed to account for the lack of inhibition of C_4 photosynthesis by oxygen levels up to 21% (2).

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**Abbreviations: C_3 plants, plants having only the enzymes of the Calvin pathway; C_4 plant, plant having enzymes of the Calvin and C_4 pathway; Chl, chlorophyll; OAA, oxalacetic acid; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; RuDP, ribulose bisphosphate.

Extracts from mesophyll protoplasts of C_4 plants fix CO_2 at high rates in the light when supplied with exogenous pyruvate, a precursor for the C_4 pathway (3,4). CO_2 fixation in these mesophyll preparations requires the conversion of pyruvate to PEP by the pyruvate Pi dikinase reaction, with a net energy requirement of 2 ATP, and the carboxylation of PEP to OAA by PEP carboxylase. We have been interested in determining whether the ATP used for CO_2 fixation in C_4 mesophyll chloroplast preparations comes from cyclic, noncyclic or pseudocyclic photophosphorylation. The contributions of cyclic and noncyclic photophosphorylation have been considered (5). In this report evidence for pseudocyclic phosphorylation (noncyclic electron flow to O_2) in C_4 mesophyll chloroplast preparations is presented.

A large contribution of pseudocyclic electron flow to CO_2 fixation in C_4 mesophyll protoplast extracts is suggested on the basis of the stimulatory effect of oxygen on pyruvate induced CO_2 fixation. In contrast, oxygen was found to inhibit the endogenous CO_2 fixation by mesophyll protoplast extracts of the C_3 plants tested.

MATERIALS AND METHODS

Mesophyll protoplasts were enzymatically isolated and purified from leaves of 2 to 3 week old plants of the C_4 plants Digitaria sanguinalis, Urochloa panicoides, and the C_3 plants Hordeum vulgare and Triticum aestivum as previously described (6,7) using the optimum conditions of Huber and Edwards (8). Mesophyll protoplast extracts, prepared by breaking the protoplasts by passage through a 20 micron nylon net, refers to chloroplasts plus cytoplasmic components (3,4). C_4 mesophyll protoplasts were broken in a medium that contained 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM Tricine-KOH (7.8) and 0.2% BSA (BSA added after breaking). With C_4 mesophyll protoplast extracts, CO_2 fixation reaction mixtures were the same except that 2 mM pyruvate (potassium salt) and $NaH^{14}CO_3$ were added. C_3 mesophyll protoplasts were broken in a mixture of 0.3 M sorbitol, 3 mM $MgCl_2$, 2 mM KH_2PO_4, 5 mM $Na_4P_2O_7$, 1 mM EDTA, 50 mM Tricine-KOH (7.6) and 0.2% BSA (BSA added after breaking). With C_3 mesophyll protoplast extracts, CO_2 fixation reaction mixtures contained 0.3 M sorbitol, 1 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM KH_2PO_4, 50 mM Tricine-KOH (8.2) and $NaH^{14}CO_3$, as indicated in the text. CO_2 fixation assays were performed in a total volume of 0.25 ml in sealed ampules that were previously gassed while shaking for 3 min. Mixtures of commercially prepared oxygen and nitrogen were used in the various oxygen experiments. After gassing, the $NaH^{14}CO_3$ was injected and reactions were initiated by the addition of the chloroplast preparations. All assays were performed at 35 C, with 80 neinsteins/$cm^2 \times s^{-1}$ of radiant energy supplied by a General Electric Lucalox sodium discharge lamp. At time intervals, 50 µl aliquots were taken and acidified, to remove unfixed $^{14}CO_2$. Rates, expressed as µmoles CO_2 fixed per mg Chl per h, were determined from the linear phase of CO_2 fixation which generally lasted 8 min.

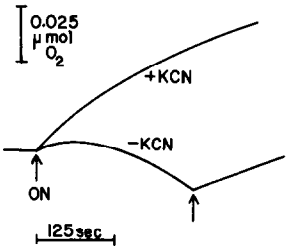

Figure 1. Polarographic trace of pyruvate-induced oxygen flux by mesophyll protoplast extracts of D. sanguinalis in the presence and absence of 2 mM KCN. Reaction mixtures were similar to those used for CO_2 fixation. Twelve μg of Chl were in a total volume of 1.5 ml. The arrow in the bottom trace indicates the addition of KCN to the control. Oxygen evolution is toward the bottom of the figure.

RESULTS

The C_4 mesophyll protoplast extracts of Digitaria sanguinalis catalyze a slow oxygen uptake in the light when supplied with pyruvate, that gradually changes from an oxygen consumption to an oxygen evolution (Fig. 1). In the presence of 2 mM KCN, a potent inhibitor of catalase, a large oxygen uptake reaction is uncovered, regardless of whether the KCN is added prior to the light period or after several minutes of illumination. The effect of cyanide suggests that oxygen is accepting electrons, perhaps from ferredoxin, to form peroxide. In another experiment, 2 mM KCN was found to have no effect on the rate of CO_2 fixation (data not shown). The effect of KCN on oxygen flux shown for D. sanguinalis was also observed with mesophyll protoplast extracts of Urochloa panicoides, another C_4 plant (data not shown).

The effect of oxygen concentration on the rate of CO_2 fixation was determined with mesophyll protoplast extracts of D. sanguinalis and H. vulgare. As shown in Fig. 2, the kinetics of $^{14}CO_2$ fixation by the C_4 mesophyll protoplast extracts of D. sanguinalis were linear with time and the rate of fixation, with respect to the rate under 2% oxygen, was increased roughly 2-fold by 21% and roughly 3-fold by 100% oxygen. As shown in Table I, the oxygen stimulated pyruvate-induced CO_2 fixation by D. sanguinalis was inhibited by antimycin A, an inhibitor of cyclic electron flow, with the greatest degree of inhibition

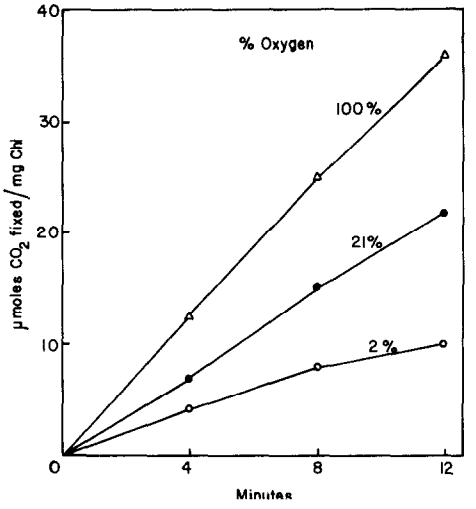

Figure 2. Effect of oxygen on the kinetics of $^{14}CO_2$ fixation by mesophyll protoplast extracts of D. sanguinalis. See Materials and Methods section for details.

Table I. Effect of oxygen on mesophyll protoplast extract $^{14}CO_2$ fixation.[a]

Species	Additions	Percent oxygen 2	21	100	Ratio (100%/2%)
		μmol/mg Chl/h			
H. vulgare (C_3)	none	24	15	11	0.46
	antimycin A	16	8.2	5.5	0.34
D. sanguinalis (C_4)	pyruvate	48	66	84	1.75
	pyruvate + antimycin A	4	26	38	9.50

[a]Bicarbonate concentration was 0.4 mM, antimycin A, 10 μM.

at low oxygen. At 2% oxygen the inhibition by antimycin A was 92% while at 100% oxygen, the inhibition decreased to 54%. The total increase in rate from 2% to 100% oxygen was roughly equivalent for pyruvate induced CO_2 fixation in the presence and absence of antimycin A. In contrast, with the C_3 mesophyll protoplast extracts of H. vulgare, oxygen inhibited CO_2 fixation (Table I).

Antimycin A inhibited CO_2 fixation roughly 45% at the different oxygen levels. Mesophyll protoplast extracts of Triticum aestivum, a C_3 plant, responded similarly (data not shown).

Another distinction between the response of C_3 and C_4 mesophyll protoplast extracts to oxygen concerns the effect of bicarbonate. As shown in Table II, oxygen stimulates pyruvate induced CO_2 fixation by the C_4 mesophyll protoplast extracts of D. sanguinalis both at rate-limiting (0.2 mM) as well as saturating (4.8 mM) levels of bicarbonate. In contrast, the inhibitory effect of oxygen on CO_2 fixation by the C_3 mesophyll protoplast extracts of H. vulgare can be reversed by high levels of bicarbonate.

DISCUSSION

The data presented suggest that in mesophyll protoplast extracts of C_4 plants, pseudocyclic electron flow can contribute significantly to the ATP required for CO_2 fixation in vitro. The relative concentrations of oxygen and NADP in vivo may control the degree of pseudocyclic versus noncyclic electron flow (5,9). With the C_4 mesophyll preparations of D. sanguinalis under 21% oxygen, KCN reveals a strong light-dependent oxygen uptake reaction by blocking catalase. Without cyanide, the peroxide formed would be rapidly broken down to $1/2\ O_2 + H_2O$ by catalase. However, if cyanide is present, a net uptake of $1/2\ O_2$ would occur for every two electrons transported from H_2O to O_2. Under low (2%) oxygen, the ATP for CO_2 fixation by the C_4 mesophyll preparations of D. sanguinalis comes primarily from cyclic phosphorylation, as antimycin A inhibited CO_2 fixation 92% (Table 1). With increasing oxygen, the rate of pyruvate-induced CO_2 fixation increased roughly 2-fold (Tables I and II). The increase observed in pyruvate-induced CO_2 fixation roughly equaled the rate under 100% oxygen in the presence of antimycin A (Table I). This increase is thought to represent the pseudocyclic contribution. Thus antimycin A, which blocks cyclic electron flow, does not affect the pseudocyclic electron flow. Also, the stimulatory effect of oxygen with the C_4 preparations was independent of bicarbonate concentration, as would be expected. Our results are in opposi-

Table II. The interaction of bicarbonate and oxygen on mesophyll protoplast extract $^{14}CO_2$ fixation.[a]

Species	$NaH^{14}CO_3$	Percent Oxygen			Ratio
		2	21	100	(100%/2%)
		μmol/mg Chl/h			
H. vulgare (C_3)	0.4 mM	24	17	11	0.46
	4.8 mM	135	137	119	0.88
D. sanguinalis (C_4)	0.2 mM	23	36	66	2.87
	4.8 mM	50	72	120	2.40

[a]See Materials and Methods section for assay details.

tion to those obtained by Chollet (10) working with mechanically isolated mesophyll cells of D. sanguinalis. He observed little effect of increasing oxygen from 2 to 21% oxygen, but found an inhibition at 100% oxygen. On the basis of his results, it was suggested that above atmospheric levels of oxygen may inhibit pyruvate - Pi dikinase. These results are difficult to interpret as the reported rates of CO_2 fixation were very low (ca. 5 μmol/mg Chl/h) relative to those in the present study. Using the conditions described in this report, mechanically isolated D. sanguinalis mesophyll cells also show an oxygen stimulation (up to 100%) of pyruvate induced CO_2 fixation (30-50 μmoles/mg Chl/h at 100% O_2; data not shown). The results reported here suggest that pyruvate-Pi dikinase is not significantly inhibited at 100% oxygen, as CO_2 fixation remained high.

In contrast to the stimulation seen with C_4 mesophyll preparations, CO_2 fixation by the C_3 mesophyll protoplast extracts of H. vulgare was inhibited by oxygen (Table I) and the inhibition could be reversed by high levels of bicarbonate (Table II). Oxygen and CO_2 are thought to be competing substrates for the enzyme RuDP carboxylase-oxygenase with oxygen reaction leading to oxygen inhibition of C_3 photosynthesis (11,12).

The C_4 chloroplasts in our extracts have been judged to be 80-90% intact on the basis of retention of chloroplast enzymes (4) and ferricyanide reduction before and after osmotic shock (Ku and Edwards, unpublished). Also in our assays, no exogenous autooxidizable cofactors (e.g., methylviologen) were used to catalyze pseudocyclic electron flow. To our knowledge, this is the first evidence for active pseudocyclic electron flow in intact chloroplasts.

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