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Oxaloacetate as the Hill Oxidant in Mesophyll Cells of Plants Possessing the C_4-Dicarboxylic Acid Cycle of Leaf Photosynthesis

(CO_2 fixation/uncoupler/photochemical reactions/electron transport/O_2 evolution)

MARVIN L. SALIN, WILBUR H. CAMPBELL, AND CLANTON C. BLACK, JR.

Department of Biochemistry, University of Georgia, Athens, Ga. 30602

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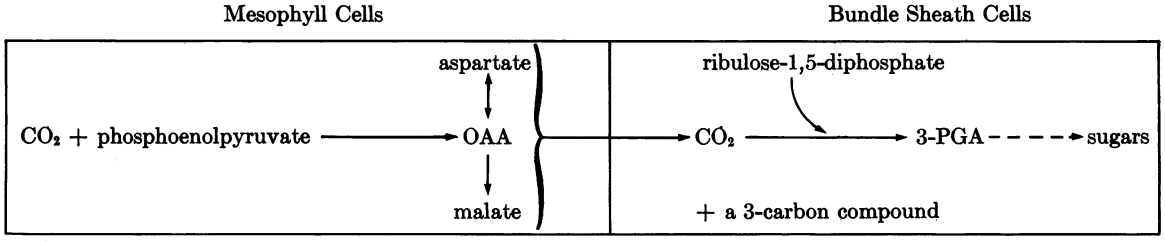
ABSTRACT **Isolated mesophyll cells from leaves of plants that use the C_4 dicarboxylic acid pathway of CO_2 fixation have been used to demonstrate that oxaloacetic acid reduction to malic acid is coupled to the photochemical evolution of oxygen through the presumed production of NADPH. The major acid-stable product of light-dependent CO_2 fixation is shown to be malic acid. In the presence of phosphoenolpyruvate and bicarbonate the stoichiometry of CO_2 fixation into acid-stable products to O_2 evolution is shown to be near 1.0. Thus oxaloacetic acid acts directly as the Hill oxidant in mesophyll cell chloroplasts. The experiments are taken as a firm demonstration that the C_4 dicarboxylic acid cycle of photosynthesis is the major pathway for the fixation of CO_2 in mesophyll cells of plants having this pathway.**

In photosynthesis by intact leaves of certain plants, "C_4 plants," the formation of the 4-carbon dicarboxylic acids, oxaloacetic (OAA), malic, and aspartic, as major (80–90% in 5 sec) initial products is rapidly followed by an increased formation of 3-phosphoglyceric acid (3-PGA), and ultimately sugars (1–4). Intact leaves of C_4 plants have a specific leaf cell arrangement with two major photosynthetic cell types: bundle sheath cells that tightly surround the veins and mesophyll cells loosely surrounding the bundle sheath cells (5, 6). It has been postulated that the major route of carbon flow in C_4 photosynthesis is:



To study the roles of these individual cell types in leaf photosynthesis we developed procedures for isolating mesophyll cells and bundle sheath cells or strands from C_4 plants (7–9). This work demonstrated that phosphoenolpyruvate (PEP) carboxylase is localized in the mesophyll cells and ribulose-1,5-diphosphate (RuDP) carboxylase is in the bundle sheath cells (7, 9). Furthermore, in C_4 plants such as crabgrass, malic enzyme is localized in the bundle sheath cells and the NADP$^+$-dependent malic dehydrogenase (10) is in the mesophyll cells (9, 11). Therefore, one could reason that in mesophyll cells the carboxylation of PEP should be coupled to the reduction of OAA and the required reduced pyridine nucleotide should be produced photosynthetically as follows:

$$HCO_3^- + PEP \xrightarrow[\text{carboxylase}]{\text{PEP}} OAA + P_i \quad [1]$$

$$NADP^+ + H_2O \xrightarrow[\text{mesophyll cell chloroplasts}]{\text{light}} NADPH + {}^1/_2\,O_2 + H^+ \quad [2]$$

$$OAA + NADPH + H^+ \xrightarrow[\text{dehydrogenase}]{\text{malate}} \text{malate} + NADP^+ \quad [3]$$

$$\text{Net: } H_2O + HCO_3^- + PEP \xrightarrow[\text{mesophyll cell}]{\text{light}} \text{malate} + {}^1/_2\,O_2 + P_i \quad [4]$$

This paper reports the photosynthetic evolution of oxygen that is dependent upon either OAA or PEP and HCO_3^- as the Hill oxidant in mesophyll cells isolated from mature leaves of C_4 plants.

MATERIALS AND METHODS

Mature leaves of *Digitaria sanguinalis* (L.) Scop. (crabgrass) and *Digitaria decumbens* (pangolagrass) were freshly harvested from greenhouse- or field-grown plants. The other *Digitaria* species in Table 1 were field-grown in Experiment, Ga. Mesophyll cells and bundle sheath strands were separated by grinding and filtration methods developed previously (8, 9).

Oxygen Evolution. Cells were added to a thermoregulated chamber fitted with an oxygen electrode (Rank Bros.). The standard assay medium in a volume of 2 ml contained: sor-

Abbreviations: C_4, C_4-dicarboxylic acid; C_3, reductive pentose phosphate; 3-PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate; RuDP, ribulose-1,5-diphosphate; OAA, oxaloacetic acid; MA, methylamine·HCl; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; CCCP, *m*-chlorocarbonyl cyanide phenylhydrazone; salicylanilide, 5-chloro,3-(*p*-chlorophenyl)-2′,4′,5′-trichlorosalicylanilide; chl, chlorophyll.

TABLE 1. *Comparison of O_2 evolution with preparations from various C_4 plants using specific Hill oxidants*

Plant	Tissue or cell type	OAA − MA	OAA + MA	PEP + HCO_3^- − MA	PEP + HCO_3^- + MA
		μmol of O_2 evolved per mg of chl per hr			
Digitaria sanguinalis (crabgrass)	Mesophyll cells	43	140	39	180
	Bundle sheath strands	0	0	0	0
Digitaria decumbens (pangolagrass)	Mesophyll cells	26	75	—	—
	Bundle sheath strands	0	0	—	—
Digitaria setivalva	Mesophyll cells	45	90	47	80
Digitaria pentzii	Mesophyll cells	16	44	16	44
Digitaria milanjiana	Mesophyll cells	23	68	18	63
Digitaria eriantha	Mesophyll cells	28	47	28	48

Cell isolation procedures and the experimental conditions were as in *Methods*. Hill oxidants were added at 5 mM.

bitol, 330 mM; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (HEPES) buffer (pH 7.5), 50 mM; OAA, 5 mM, or PEP, 5 mM; where indicated, methylamine·HCl(MA), 15 mM, CCCP, DCMU, and salicylanilide, 20 mM; plus 0.1–0.2 ml of cells in isolation medium usually containing 10–20 μg of chlorophyll. In the standard assay medium sufficient HCO_3^- was dissolved in the medium for the carboxylation of PEP to occur.

$NaH^{14}CO_3$ Fixation by Mesophyll Cells was studied as previously described (7, 11). The reaction products of all light and dark $H^{14}CO_3^-$ fixation experiments were chromatographed two-dimensionally using standard procedures (12).

To measure total fixation of $H^{14}CO_3^-$, the reaction was stopped by mixing in a scintillation vial an aliquot of the reaction mixture with an equal volume either of 0.2% phenylhydrazine·HCl (w/v) in 2 N HCl or of 88% formic acid. Gaseous $^{14}CO_2$ was removed from the vials by blowing with a stream of compressed air for 1 min. The radioactivity of the samples was determined in a Packard Tri-Carb liquid scintillation spectrophotometer (model 2002). For measurement of fixation of $H^{14}CO_3^-$ into products which were stable to heat treatment in an acid medium, a 50-μl aliquot of the reaction mixture was mixed with 0.2 ml of 88% formic acid and heated in an oven at 80° for 1 hr. This treatment effectively removes unreacted $H^{14}CO_3^-$, $^{14}CO_2$, and ^{14}C fixed as carbon-4 of OAA. Acid-stable ^{14}C-labeled products were identified chromatographically.

Chlorophyll (chl) was determined in a 96% ethanol solution with the absorption coefficients of Wintermans and De Mots (13).

RESULTS AND DISCUSSION

Oxygen Evolution by Mesophyll Cells. Previous electron transport work with isolated crabgrass leaf cells has shown that mesophyll cells photosynthetically evolve O_2 when artificial Hill oxidants such as benzoquinone are added to cell suspensions (14). Hence the purpose of this research is to examine photosynthetic reactions in mesophyll cells from C_4 plant leaves using naturally occurring Hill oxidants. Since CO_2 in the mesophyll cells is fixed primarily by PEP carboxylase (reaction [1]), we began by adding OAA to cell suspensions. Fig. 1*A* and *B* show traces from typical chart recordings of the general pattern of O_2 metabolism observed with crabgrass mesophyll cells. In Fig. 1*A* when OAA was mixed with mesophyll cells, O_2 was evolved. The rate of O_2 evolution was increased upon addition of MA, a photosynthetic uncoupler (15). The enhancement of O_2 evolution by MA was not specific, since NH_4Cl at 2 mM, CCCP at 2.5 μM, salicylanilide at 10 μM, and pyruvate at 5 mM were quite effective. The enhancement of O_2 evolution with uncouplers varied from 1.7 to 4.0 in day-to-day experiments.

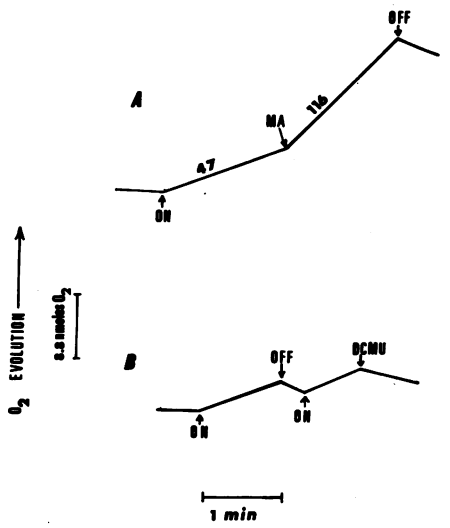


FIG. 1. (A) OAA-mediated evolution of O_2 with crabgrass mesophyll cells in coupled and uncoupled state. *On* and *off* indicate the beginning and termination of illumination; *MA*, the addition of 15 mM methylamine·HCl. The numbers refer to μmol of O_2 evolved per mg of chl per hr; chl conc., 10 μg. (B) OAA-mediated O_2 evolution with mesophyll cells in light, dark, and in the presence of DCMU. Conditions identical to those in (A); DCMU concentration, 20 μM.

The inhibitor DCMU caused O_2 evolution to cease completely (Fig. 1*B*). O_2 Evolution also ceased in darkness (*OFF* in Fig. 1*A* and *B*) and was replaced by a rapid O_2 uptake which slowly declined to the dark level. A similar unexplained dark O_2 uptake pattern has been reported with chloroplasts isolated from a C_3 plant (16). It should be noted that illuminated cells without substrate will take up O_2 in a Mehler-type reaction (17). This O_2 uptake activity has not been investigated, but it is light-dependent and occurs at a rate between 15 and 25 μmole/mg of chl per hr in day-to-day experiments. However, it is insensitive to boiling or heating cells for 5 min at 50°, which will destroy the O_2 evolution activities shown in Fig. 1*A* and *B* and the CO_2-fixation reactions presented later.

The optimum experimental conditions for studying oxygen evolution were examined and the results are presented in Fig. 2. The temperature optimum was near 40° with OAA as the Hill oxidant and near 35° with PEP and HCO_3^- as the Hill oxidant (Fig. 2*A*). We chose 35° as the standard temperature for further work. This temperature compares favorably with the optimum range for C_4 photosynthesis (4). The pH optimum in the uncoupled system was near 7.5, but the

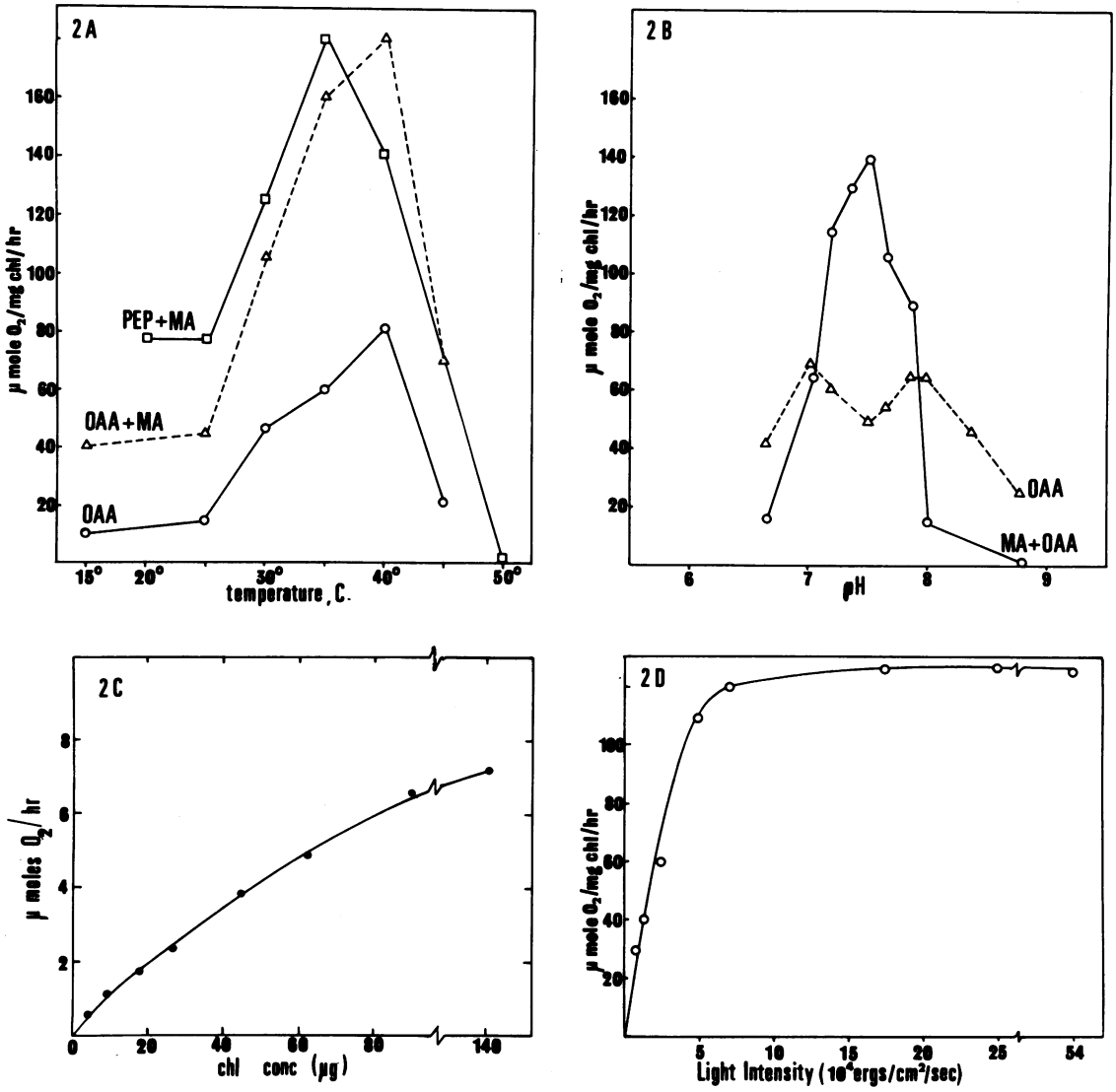


FIG. 2. Influence of experimental conditions upon O_2 evolution with isolated crabgrass mesophyll cells. The standard concentrations of reaction components used are given in *Materials and Methods*. (*A*) Influence of temperature on PEP- and OAA- mediated O_2 evolution. (*B*) Influence of pH on OAA- and PEP-mediated O_2 evolution in coupled and uncoupled states; HEPES buffer. (*C*) Influence of chlorophyll concentration on O_2 evolution with OAA as the Hill oxidant. (*D*) Influence of illumination intensity with OAA as the Hill oxidant.

coupled system exhibited an unexplained double peak (Fig. 2*B*). Hill and Walker (18) observed a similar double peak for photophosphorylation using C_3 chloroplasts. We chose pH 7.5 for subsequent work. O_2 evolution increased hyperbolically with increasing cell concentration (Fig. 2*C*), and we worked in the range of 10–20 μg of chlorophyll per reaction mixture. Light saturation was obtained near 10^5 ergs/cm² per sec (Fig. 2*D*) and subsequently 1.5×10^5 ergs/cm² per sec was employed. The isolated mesophyll cells, stored in ice at concentrations near 0.3–0.4 mg of chl per ml, maintained their ability to evolve O_2 over a 1- to 2-hr period with no substantial loss in specific activity.

If OAA was replaced by PEP plus HCO_3^-, quite similar O_2 evolution results were obtained. Only small differences (0–15% maximum) were noted in the specific activities of O_2 evolution with OAA versus PEP and HCO_3^- as the respective Hill oxidant in day-to-day experiments. Apparent K_m values for OAA and PEP as Hill oxidants were 0.31 mM and 0.67 mM, respectively.

Since the use of PEP as a Hill oxidant involves a carboxylation (reaction [1]), the O_2 evolution should be dependent upon HCO_3^-. When reaction mixtures containing PEP were treated to remove as much CO_2 and HCO_3^- as possible and illuminated as in Fig. 3, the O_2 evolution terminated when the CO_2 and HCO_3^- supply was depleted. Upon the addition of HCO_3^-, the O_2 evolution immediately resumed (Fig. 3). Clearly, O_2 evolution with PEP as the Hill oxidant is dependent upon CO_2-fixation in these mesophyll cells.

Similar experiments using OAA or PEP and HCO_3^- as Hill oxidants were attempted with isolated bundle sheath strands from crabgrass and pangolagrass (Table 1) and no O_2 evolution was observed. In total we have examined O_2 evolution in six C_4 species and found the isolated mesophyll cells from all species to be similar to those of crabgrass in exhibiting a light-dependent O_2 evolution in the presence of OAA or PEP and HCO_3^- (Table 1). Thus, our experiments indicate that mesophyll cells from several C_4 plants evolve O_2 in the presence of OAA and directly couple O_2 evolution to CO_2

fixation. These observations further suggest that the CO_2 fixation is coupled to O_2 evolution through reduction of OAA.

$H^{14}CO_3^-$ Fixation by Isolated Mesophyll Cells. When supplied with PEP, the mesophyll cells of crabgrass (and other C_4 plants) rapidly fix $H^{14}CO_3^-$ into dicarboxylic acids in the light or dark (refs. 9 and 11; unpublished data). The specific activity for fixation is similar in the light or the dark and under optimal conditions V_{max} can be as high as 2000 μmol of $H^{14}CO_3^-$ fixed per mg of chl per hr. The apparent Michaelis constants for this reaction were found to be similar in the light and the dark. The K_ms at 30° are: PEP, 1.5 mM; $H^{14}CO_3^-$, 1.5 mM; and $MgCl_2$, 1.1 mM.

In the dark over 97% of the ^{14}C was found in OAA, extracted as the phenylhydrazone, whereas in the light 87.5% of the radioactivity was found in OAA. The other products formed in the light were found to be malic and aspartic acids. After 2 min of fixation, malic acid was 10.1% of the total ^{14}C fixed and aspartic acid 2.2%. Since malic and aspartic acids are only formed in the light, the appearance of these products was probably coupled to the photosynthetic process through the reduction of OAA, reaction [3].

Since carbon-4 of OAA is rapidly cleaved when OAA is heated in an acidic medium, the rate of formation of other products of the light-dependent reduction of OAA could be studied. Therefore, we examined the light-mediated incorporation of ^{14}C into malic and aspartic acids to determine the relationship between oxygen evolution and appearance of ^{14}C in acid-stable C_4 dicarboxylic acids.

In Fig. 4, the results of $H^{14}CO_3^-$ fixation experiments with mesophyll cells from crabgrass are shown. In curve *A* the rate of $H^{14}CO_3^-$ fixation in the presence of excess PEP into all products was shown to be independent of the photosynthetic uncouplers, MA and CCCP, and of the inhibitor DCMU. As was previously shown (7), the fixation continued unaffected in the transition from light to dark (curve *A*). Clearly the carboxylation reaction to form OAA is not influenced by light, dark, inhibitors, or uncouplers. In experiments where the labeled OAA was removed by heating in acid, essentially no radioactivity was incorporated in the dark (curve *A*). However, in the light, ^{14}C was incorporated into acid-stable products and the rate of $H^{14}CO_3^-$ fixation into malic and aspartic acid (the acid-stable products) was stimulated by addition of MA or CCCP (curves *B* and *C*). Thus, uncouplers stimulated CO_2-fixation in a fashion similar to the oxygen evolution reaction described previously. $H^{14}CO_3^-$ fixation into malic and aspartic acid was stopped either by the addition of DCMU or by turning off the light (curves *B* and *C*). No fixation of $H^{14}CO_3^-$ into malic acid or aspartic acid was observed in dark controls or if DCMU was added before the light was turned on. In day-to-day experiments with PEP plus MA the light-dependent incorporation of $H^{14}CO_3^-$ into products other than OAA varied between 150 and 315 μmol of $H^{14}CO_3^-$ fixed per mg of chl per hr with crabgrass mesophyll cells.

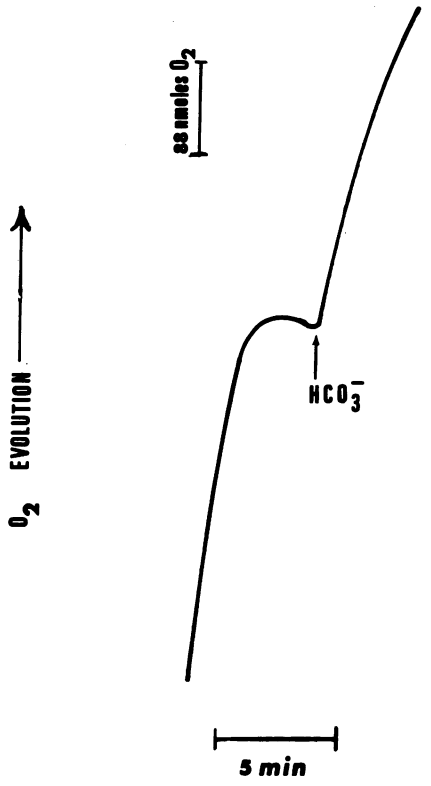


FIG. 3. HCO_3^- dependence of O_2 evolution by isolated crabgrass mesophyll cells with PEP as the Hill oxidant. The reaction buffer was bubbled for 10 min with nitrogen and the reaction mixture with cells was gassed for 1 min to remove CO_2 and HCO_3^-. PEP was added and the reaction mixture was illuminated to allow the PEP carboxylase to consume the residual HCO_3^-. When O_2 evolution ceased, 10 mM HCO_3^- was added at the *arrow*, and O_2 evolution immediately resumed. Chl conc., 80 μg.

The Relationship of O_2 Evolution to [^{14}C]Malic Acid Synthesis in the Light. When the specific activity of oxygen evolution and the specific activity of $H^{14}CO_3^-$ fixation into malic acid (and aspartic acid) by mesophyll cells in the light were compared, similar activities were found. Table 2 shows a comparison of specific activities obtained for coupled and

TABLE 2. *Stoichiometry of light-dependent $NaH^{14}CO_3$ fixation into acid-stable products to O_2 evolution with isolated mesophyll cells*

	Coupled			Uncoupled		
Exp. no.	μmol of O_2/mg of chl per hr	μmol of $H^{14}CO_3^-$ fixed/mg of chl per hr	μmol of $H^{14}CO_3^-$ / μatoms O_2	μmol O_2/mg of chl per hr	μmol of $H^{14}CO_3^-$ fixed/mg of chl per hr	μmol of $H^{14}CO_3^-$ / μatoms O_2
1	36	67	0.93	79	153	0.98
2	28	60	1.07	89	165	0.93
3	—	—	—	36	98	1.36

Absolute rates of O_2 evolution and $NaH^{14}CO_3$ fixation into acid-stable products were determined at 35°, according to procedures outlined in *Methods*. In experiments 1 and 2, 15 mM MA was used as an uncoupler; in experiment 3, 20 μM CCCP was employed.

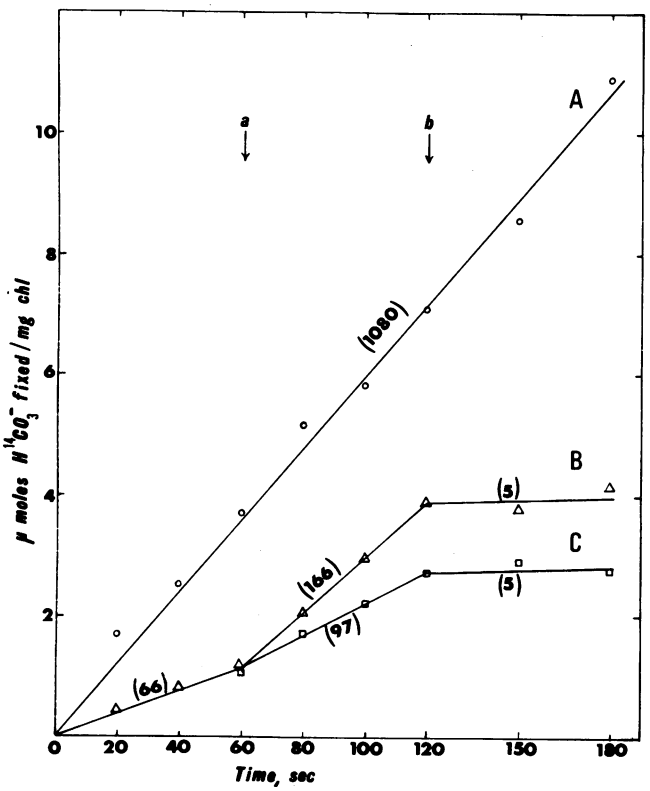


FIG. 4. $H^{14}CO_3^-$ fixation by isolated crabgrass mesophyll cells in the presence of PEP. The reactions were run at pH 7.5 and 35° at a light intensity of 1.5 × 10^5 ergs/cm² per sec when illuminated. *A*. $H^{14}CO_3^-$ fixation into all products in light or dark. Curve *A* is presented at 20% of the actual rate. *B* and *C*. $H^{14}CO_3^-$ fixation into acid- and heat-stable products. *Arrow 'a'* indicates the time when an uncoupler was added; either MA or CCCP for curve *A;* MA for curve *B;* or CCCP for curve *C*. *Arrow 'b'* indicates the time when DCMU was added or the light was turned off; either DCMU or light off for curve *A;* DCMU for curve *B;* or light off for curve *C*. The concentrations of all compounds added are described in *Materials and Methods*. Specific activities shown in parentheses are in μmol of $H^{14}CO_3^-$ fixed per mg of chl per hr.

uncoupled reactions in the same preparation of mesophyll cells. The ratios of μmol of $H^{14}CO_3^-$ fixed into acid-stable products to μatoms of O_2 evolved were near 1.0 (Table 2). These data suggest a close relationship between the rate of O_2 evolution, a chloroplast photochemical process, and the rate of malic acid synthesis resulting from $H^{14}CO_3^-$ fixations.

CONCLUSIONS

Photosynthetic evolution of O_2 by isolated chloroplasts in the presence of an artificial electron acceptor, ferric oxalate, was first demonstrated by Hill (19). In the C_3 cycle of photosynthetic CO_2 fixation (12) the natural electron acceptor (Hill oxidant) is 1,3-diphosphoglyceric acid that is reduced by the NADPH produced photosynthetically. Walker and coworkers (16, 20) have shown that various isolated chloroplast preparations from C_3 plants evolve O_2 in the presence of C_3 carbon cycle intermediates such as 3-PGA.

When whole leaf or whole plant photosynthesis in C_4 plants is considered, the rates of photosynthetic O_2 evolution or CO_2 uptake range between 200 and 400 μmol of O_2 evolved or of CO_2 taken up per mg of chl per hr (4). Thus, the O_2 evolution and CO_2 fixation rates reported in these experiments with isolated leaf mesophyll cells are comparable with whole leaf and whole plant studies.

Despite the fact that numerous workers have reported the initial formation of dicarboxylic acids in C_4 plant leaf experiments (3, 4), it has not been demonstrated that the CO_2 fixation reaction (reaction [1]) to form dicarboxylic acids can be directly coupled to the photochemical reactions of C_4 photosynthesis. However, the mesophyll cell chloroplasts have been conclusively demonstrated to contain the usual components of Photosystems I and II (14). The dependence of O_2 evolution upon HCO_3^-, shown in Fig. 3, is a direct demonstration of the coupling of photochemical reactions to CO_2 fixation in C_4 mesophyll cells. Furthermore, the demonstration that the primary acid-stable product of this light-dependent CO_2 fixation is malic acid (Fig. 4 and $H^{14}CO_3^-$ fixation results) is taken as evidence that NADPH was produced photochemically as in reaction [2] and subsequently utilized by malic dehydrogenase (NADPH) as in reaction [3]. If the O_2 evolution studies, Fig. 1*A* and *B*, are compared with the CO_2 fixation studies, Fig. 4*B* and *C*, both activities are affected in an identical fashion by light, uncouplers, or an electron transport inhibitor. Finally, the net reaction (reaction [4]) shows that the theoretical stoichiometry of μmol of CO_2 fixed to μatoms of O_2 evolved should be 1 and values near 1 were presented in Table 2. These experiments are taken as a firm demonstration that the C_4 cycle of photosynthesis is the major pathway for the fixation of CO_2 in mesophyll cells of C_4 plants.

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