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C_4 Photosynthesis: Light-dependent CO_2 Fixation by Mesophyll Cells, Protoplasts, and Protoplast Extracts of *Digitaria sanguinalis*[1]

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ABSTRACT

Mesophyll cells, protoplasts, and protoplast extracts of *Digitaria sanguinalis* were used for comparative studies of light-dependent CO_2 fixation. CO_2 fixation was low without the addition of organic substrates. Pyruvate, oxaloacetate, and 3-phosphoglycerate induced relatively low rates (10 to 90 μmoles/mg chlorophyll·hr) of CO_2 fixation when added separately. However, a highly synergistic relationship was found between pyruvate + oxaloacetate and pyruvate + 3-phosphoglycerate for inducing light-dependent CO_2 fixation in the mesophyll preparations. Highest rates of CO_2 fixation were obtained with protoplast extracts. Pyruvate, in combination with oxaloacetate or 3-phosphoglycerate induced light-dependent rates from 150 to 380 μmoles of CO_2 fixed/mg chlorophyll·hr which are equivalent to or exceed reported rates of whole leaf photosynthesis in C_4 species. Concentrations of various substrates required to give half-maximum velocities of CO_2 fixation were determined, with the protoplast extracts generally saturating at the lowest substrate concentrations. Chloroplasts separated from protoplast extracts showed little capacity for CO_2 fixation. The results suggest that CO_2 fixation in C_4 mesophyll cells is dependent on chloroplasts and extrachloroplastic phosphoenolpyruvate carboxylase.

The stimulation of pyruvate-induced CO_2 fixation by oxaloacetate and 3-phosphoglycerate is thought to be due to induction of noncyclic electron transport which generates ATP for the conversion of pyruvate to phosphoenolpyruvate by pyruvate Pi dikinase. The primary products of the substrate-induced CO_2 fixation were oxaloacetate and malate, which provides further evidence for carbon fixation through the β-carboxylation pathway. High rates of light-dependent CO_2 fixation with a significant percentage of ^{14}C fixed into malate suggest an efficient operation of both photosystems I and II.

The substrate inductions are discussed with respect to the proposed role of the mesophyll cell in C_4 photosynthesis, and schemes suggesting the stoichiometry of energy requirements for photosynthetic carbon metabolism in C_4 mesophyll cells are presented.

In C_4 plants, high efficiency CO_2 fixation is thought to require the coordinated functioning of the two chlorophyllous cell types: the mesophyll and bundle sheath cells. It is proposed that atmospheric CO_2 is initially fixed in the mesophyll cells by PEP[2] carboxylase with the formation of OAA and subsequently malate and aspartate. The C_4 acids would be transported to the bundle sheath and decarboxylated, forming a concentrated CO_2 pool for subsequent refixation via the Calvin pathway (8, 13).

Much of what is known about the intercellular operation of the C_4 pathway has come from studying the distribution of enzymes between the two cell types (4, 5, 7–10, 12, 24–26). The argument still persists, however, that enzyme distributions do not establish a metabolic pathway. For studying the photosynthetic functions of the two cell types, either isolated chloroplasts or cells can be used. Bundle sheath cells enzymatically isolated from a number of C_4 species fix CO_2 without added organic substrates at rates which correlate with the level of photosystem II (27, 29). The products of bundle sheath CO_2 fixation suggest carbon assimilation by the Calvin pathway (8, 21, 37, Huber and Edwards, unpublished data). *In vivo*, C_4 acids would serve as the primary source of CO_2 for the Calvin pathway in bundle sheath cells, a proposal supported by the capacity of bundle sheath cells of various C_4 species to decarboxylate malate at rates equivalent to the rate of whole leaf photosynthesis (19) and evidence that malate can serve as a carboxyl donor to the Calvin pathway (21).

In species such as maize, sugarcane, and *Digitaria sanguinalis*, pyruvate—a product of malate decarboxylation by NADP-malic enzyme in bundle sheath cells—is proposed to be transported from the bundle sheath to the mesophyll cells and converted to PEP by pyruvate Pi dikinase. The PEP thus formed is the substrate for the β-carboxylation in the mesophyll cells. The operation of a β-carboxylation pathway in C_4 mesophyll cells is supported by localization of PEP carboxylase, pyruvate Pi dikinase, and certain other enzymes in mesophyll cells (5, 12, 15, 24, 25) and the ability of C_4 mesophyll chloroplasts to convert pyruvate to PEP (22).

However, attempts to demonstrate pyruvate-induced CO_2 fixation in isolated mesophyll cells or chloroplasts have provided low rates (6, 10), and doubts have been raised as to the functional role of C_4 mesophyll cells *in vivo* (30).

This paper reports conditions required for high rates of pyruvate-dependent CO_2 fixation with isolated mesophyll cells, mesophyll protoplasts, and protoplast extracts of *D. sanguinalis*. *D. sanguinalis* is a unique plant in that methods have been devised mechanically to isolate mesophyll cells and enzymatically to isolate mesophyll protoplasts, from which photochemically functional chloroplasts can be isolated. Conse-

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[2] Abbreviations: PEP: phosphoenolpyruvate; OAA: oxaloacetate; PGA: 3-phosphoglycerate.

quently, this species provides an interesting system for studying the comparative metabolism of C_4 mesophyll cells, protoplasts, and chloroplasts.

MATERIALS AND METHODS

PLANT CULTURE

Plants were grown in a growth chamber under 16 hr light and 8 hr dark, with a day temperature of 30 C and a night temperature of 25 C. Light was provided by a combination of incandescent and fluorescent lamps giving a total quantum flux density of 50 to 70 nanoeinsteins·cm^{-2} sec^{-1} between wavelengths of 400 to 700 nm. Leaves were collected from plants 2 to 4 weeks old.

ISOLATION TECHNIQUES

Mesophyll Cells. Mesophyll cells were isolated by the grinding and filtration method of Edwards and Black (7).

Mesophyll Protoplasts. Mesophyll protoplasts were enzymically isolated by the method of Kanai and Edwards (24, 25) using optimum conditions described by Huber and Edwards (in preparation).

Protoplast Extracts. Mesophyll chloroplasts were studied using the total protoplast extract obtained from mesophyll protoplasts by passage through a 20-μm nylon net. Previous experiments on intracellular localization of enzymes indicated that chloroplasts remain largely intact when extracted by this method (11). In the present study, the chloroplast preparations studied also contained all extrachloroplastic enzymes and are therefore referred to as protoplast extracts. For breakage, mesophyll protoplasts were adjusted to a Chl concentration of about 200 μg Chl/ml and were broken in a medium that contained: 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 2 mM KH_2PO_4, and 50 mM Tricine-KOH adjusted to pH 7.5 (breaking medium). Immediately after breaking BSA was added to give a final concentration of 0.5 mg/ml. Reducing agents, such as dithiothreitol, were not necessary and, in general, were inhibitory when included in either the breaking medium or reaction mixture. All preparations were made and maintained at room temperature and protoplast extracts were used immediately after preparation.

CO_2 FIXATION ASSAYS

All CO_2 fixation assays were performed in reaction mixtures that contained: 0.3 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM EDTA, 2 mM KH_2PO_4, 50 mM Tricine-KOH (pH 7.5), and 6 mM NaH $^{14}CO_3$. Unless indicated otherwise in specific experiments, substrate concentrations were 0.5 mM OAA, 5 mM pyruvate, 1 mM PGA, 2 mM α-ketoglutarate, and 0.5 mM NH_4Cl. All assays were performed in a total volume of 250 μl in round bottom test tubes. The reaction tubes were incubated —without shaking in a water bath—at 40 C, which was found to be optimum. Except as indicated, the assays were performed in the light, with a total quantum flux of about 80 nanoeinsteins cm^{-2} sec^{-1} between 400 and 700 nm provided by a General Electric sodium discharge lamp. At various intervals, 40-μl aliquots were removed and the incorporation of $^{14}CO_2$ into acid-stable products was determined by liquid scintillation counting. For determining HCO_3^- saturation curves, reactions were run under nitrogen in sealed ampules with varying concentrations of $NaH^{14}CO_3$. Rates of CO_2 fixation are expressed as μmoles/mg Chl·hr.

O_2 EVOLUTION

Photosynthetic O_2 evolution was measured polarographically in a water-jacketed Plexiglas chamber maintained at 30 C as previously described (27). The assay solution, the same as that used for CO_2 fixation studies, was agitated by a magnetic stirrer. Light was provided by a 150-w General Electric projector flood lamp giving a quantum flux density of roughly 120 nanoeinsteins cm^{-2} sec^{-1} between 400 and 700 nm at the surface of the cuvette.

CHROMATOGRAPHY OF END-PRODUCTS

The products of protoplast extract fixation were determined after 10 min of $^{14}CO_2$ fixation by killing the reaction mixture with 1 ml of 0.2% 2,4-dinitrophenylhydrazine in 2 N HCl. After 30 min, the phenylhydrazone derivatives were extracted into 2 ml of chloroform-ethanol (4:1). The organic phase containing the phenylhydrazone derivatives was concentrated and chromatogramed on one-dimensional strips in butanol-ethanol-0.5 N NH_4OH (7:1:2, v/v). Components of the aqueous phase were resolved by two-dimensional chromatography in phenol-water-acetic acid (73:22:1) with 1 mM EDTA, and butanol-propionic acid-water (105:50:71) followed by autoradiography.

CHLOROPHYLL DETERMINATION

Chlorophyll was determined by the method of Wintermans and De Mots (40).

RESULTS

Effects of Substrates. In general, mesophyll cells, protoplasts, and protoplast extracts of *Digitaria sanguinalis* responded in a similar fashion to substrate induction of CO_2 fixation although quantitatively protoplast extracts gave the highest rates. Typical results are shown in Table I in which all treatments were performed on the same isolations. There was little CO_2 fixation with mesophyll cells, protoplasts, or protoplast extracts, without added substrates. Pyruvate, OAA, and PGA, when added separately induced relatively low rates of CO_2 fixation. The pyruvate induction and OAA induction were light-dependent (dark activity not shown) while there was some fixation in the dark with PGA. High rates of CO_2 fixation of the same magnitude were induced when either pyruvate + OAA or pyruvate + PGA were added. The combination of pyruvate + OAA + PGA induced slightly higher rates than either pyruvate + OAA or pyruvate + PGA. The induction of CO_2 fixation by pyruvate + OAA was entirely light-dependent, while the induction by pyruvate + OAA + PGA was largely light-dependent. High rates of CO_2 fixation with the protoplast extracts were largely dependent on the presence of the supernatant, since the rates were low (23% of the control) if chloroplasts were separated from the supernatant by centrifugation.

Addition of α-ketoglutarate and NH_4^+ did not stimulate the pyruvate-induced CO_2 fixation as did OAA. NH_4^+ at 0.5 mM did not affect the rate induced by pyruvate + OAA with protoplasts or protoplast extracts, whereas it caused a reduction in fixation by mesophyll cells.

The addition of PEP at a concentration of 1 mM induced high rates of CO_2 fixation in mesophyll cells and protoplasts in both light and dark with slightly higher rates in the light. Ribose-5-P and ribose-5-P + OAA were checked for induction and found not to induce significantly with either of the preparations.

Table I. *Effect of Various Substrates on CO_2 Fixation by Isolated Mesophyll Cells, Protoplasts, and Protoplast Extracts of Digitaria sanguinalis*

Substrate concentrations were: 10 mM pyruvate, 0.5 mM OAA, 1 mM PGA, 0.5 mM NH_4Cl, 2 mM α-ketoglutarate, and 5 mM R5P (ribose 5-phosphate). Rates were calculated from the linear phase of CO_2 fixation which usually lasted at least 10 min.

Addition	Rate		
	Mesophyll cells	Mesophyll protoplasts	Protoplast extract
	μmoles/mg Chl·hr		
None, light	<1	<1	<1
Pyruvate, light	10	16	40
OAA, light	6	9	14
Pyruvate + OAA, light	46	52	253
Pyruvate + OAA, dark	3	<1	<1
PGA, light	23	23	97
PGA, dark	12	8	92
Pyruvate + PGA, light	36	63	313
Pyruvate + OAA + PGA, light	70	89	380
Pyruvate + OAA + PGA, dark	19	6	97
Pyruvate + OAA + NH_4Cl, light	13	47	220
Pyruvate + α-ketoglutarate + NH_4Cl, light	6	ND[1]	37
1 mM PEP, light	305	465	ND
1 mM PEP, dark	232	249	ND
10 mM PEP, light	996	870	ND
R5P, light	3	2	2
R5P + OAA, light	9	ND	14

[1] Not determined.

Typical kinetics of CO_2 fixation by mesophyll cells, protoplasts, and protoplast extracts are shown in Figure 1. When mesophyll cells are supplied with either pyruvate or OAA, light-dependent CO_2 fixation is low; however, when supplied with pyruvate + OAA, CO_2 fixation is enhanced and becomes linear with time up to 30 min (Fig. 1a). Mesophyll protoplasts, unlike mesophyll cells, exhibited a time lag of 4 to 6 min before maximum CO_2 fixation occurred (Fig. 1b). After the initial time lag, fixation proceeded linearly when the protoplasts were supplied with pyruvate + OAA, or pyruvate + OAA + PGA. The time lag of CO_2 fixation which was often seen with mesophyll protoplasts was unaffected by a 5-min preincubation in the light (data not shown). When only pyruvate was added, fixation was relatively low.

In contrast to mesophyll cells and protoplasts which, when supplied with pyruvate + OAA fixed CO_2 linearly for at least 20 min, protoplast extracts under optimum conditions fixed CO_2 linearly up to about 12 min (Fig. 1c).

Composition of Reaction Mixture. Parameters of the reaction mixture, such as pH, salts, and the osmoticum, were checked for maximum induction when pyruvate + OAA were added. A strong pH dependence of CO_2 fixation was found with mesophyll cells, whereas the fixation by mesophyll protoplasts and protoplast extracts exhibited nearly identical pH curves, with a broad peak of activity centered at pH 7.5 (Fig. 2). Other experiments were performed at pH 7.5.

The addition of Pi and Mg^{2+} was found to be important for maximum rates with the three mesophyll isolations (Fig. 3). As shown in Figure 3a, 1 mM Pi was saturating for all three preparations. Double-reciprocal plots, however, yielded apparent *Km* values for Pi of 0.11 and 0.54 mM for mesophyll cells and protoplasts, respectively. It was difficult to determine the *Km* of Pi for mesophyll chloroplast preparations because Pi was absolutely required in the breaking medium, and the addition of the chloroplast suspension to the reaction medium indirectly added Pi. However, the *Km* for Pi with the mesophyll chloroplast preparation is clearly less than 0.1 mM. Two mM Pi was routinely included in other assays. CO_2 fixation by mesophyll protoplast extracts also saturated at lower concentrations of Mg^{2+} than did mesophyll protoplasts and cells (Fig.

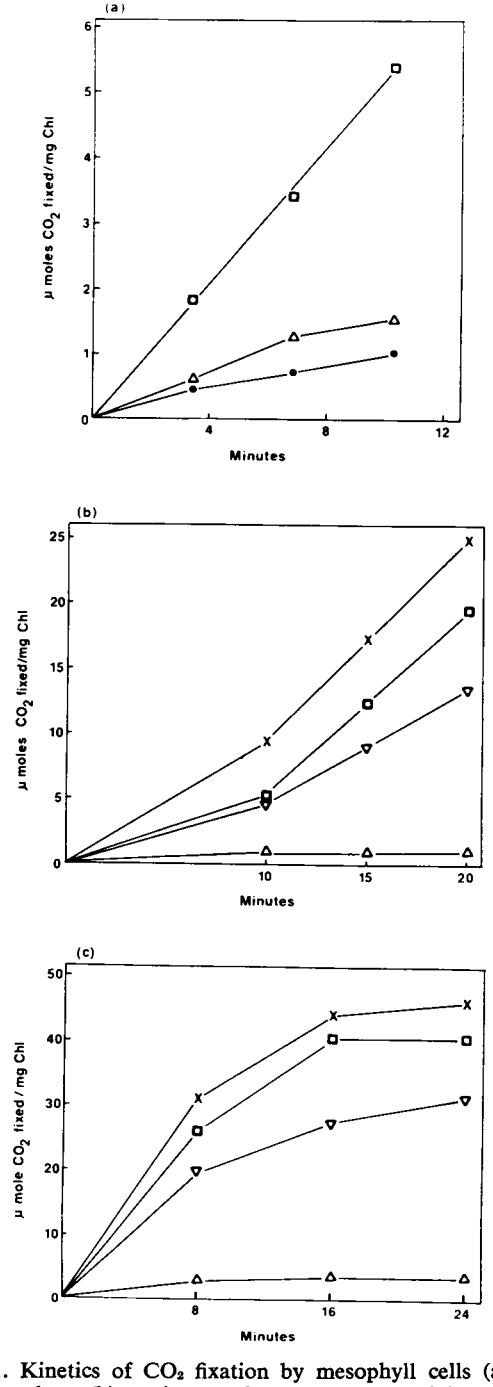

FIG. 1. Kinetics of CO_2 fixation by mesophyll cells (a), mesophyll protoplasts (b), and protoplast extracts (c) of *Digitaria sanguinalis*, with the addition of various substrates: pyruvate (△——△); OAA (●——●); pyruvate + OAA (□——□); pyruvate + PGA (▽——▽); pyruvate + OAA + PGA (×——×).

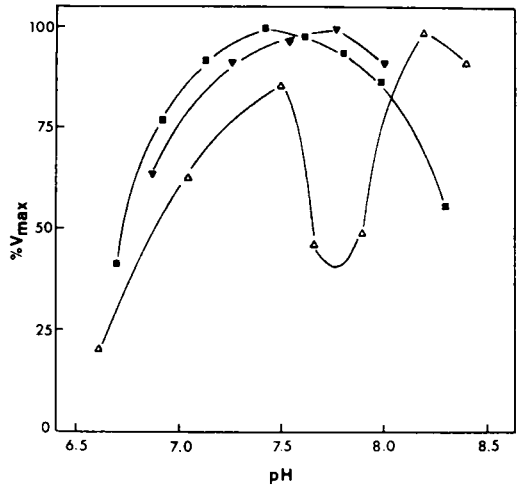

FIG. 2. Effect of pH on the rate of pyruvate + OAA dependent CO_2 fixation by mesophyll cells (△——△), mesophyll protoplasts (▼——▼), and protoplast extracts (■——■) of *Digitaria sanguinalis*. Maximum velocities attained by the cells, protoplasts and protoplast extracts were, respectively, 28, 25, and 100 μmoles/mg Chl·hr.

3b). Concentrations of $MgCl_2$ greater than 0.6 mM were inhibitory with mesophyll cells, but protoplasts and protoplast extracts were unaffected at concentrations up to 2 mM. The K*m* value for added Mg^{2+} with protoplast extracts was low but again could not be accurately determined due to an absolute requirement for Mg^{2+} in the breaking medium. Double-reciprocal plots yielded apparent K*m* values for Mg^{2+} of 0.19 and 0.11 mM for mesophyll cells and protoplasts, respectively. One mM $MgCl_2$ was used for other assays.

The concentration of sorbitol was found to be an important determinant of maximum rates of CO_2 fixation with the three mesophyll isolations. Sorbitol concentrations over the range of 0.1 to 0.7 M were checked. A sharp optimum at 0.3 M was obtained with mesophyll cells, whereas mesophyll protoplast activity continued to increase as the concentration of sorbitol was lowered below 0.3 M. However, at sorbitol concentrations less than 0.3 M, the protoplasts were subject to breakage; therefore, 0.3 M sorbitol was used routinely for cells, protoplasts, and protoplast extracts.

Saturation Characteristics of Various Substrates. In an effort to characterize the CO_2 fixation process in mesophyll cells, protoplasts, and protoplast extracts, CO_2 fixation rates were determined with varying concentrations of the substrates found to be effective inducers. In cases where substrate combinations gave a synergistic response, one of the substrates was held constant, at a rate-saturating level, while the other was varied. Since the rate of CO_2 fixation is negligible without added substrates, apparent K*m* values could be determined only when one substrate, for which there was an absolute requirement (such as pyruvate), was being added. When a synergistic response was being studied, it was possible only to determine the concentration of the varied substrate that gave half-maximum reaction velocity, inasmuch as an absolute requirement for the second substrate (such as OAA or PGA) did not exist.

From the data in Figure 4, two trends are seen: first, the apparent K*m* values or the concentrations required to give half-maximum velocity are low, and second, protoplast extracts usually are saturated before protoplasts and cells. Protoplast extracts required the lowest concentrations of substrates to give half-maximum volocity in all cases except when the concentration of pyruvate was varied in the presence of 0.5 mM OAA, where the protoplasts saturated at a lower concentration of pyruvate than did the protoplast extracts.

When the concentration of OAA or PGA was varied in the presence of saturating levels of pyruvate, OAA induced slightly higher maximum velocities at lower concentrations than did PGA with all three mesophyll preparations.

Products of CO_2 Fixation by Protoplast Extracts. The products of CO_2 fixation induced by various substrate combinations with protoplast extracts were determined after 10 min of fixation (Table II). The most striking feature of the product distribution is the high percentage of ^{14}C fixed into C_4 acids, with OAA and malate being the primary products. On a relative basis, the highest percentage of ^{14}C-OAA was formed when exogenous OAA was added. Whereas the substrate combination pyruvate + OAA gave the highest relative percentage of ^{14}C-OAA and the lowest relative percentage of ^{14}C-malate, the combination of pyruvate + PGA gave the least ^{14}C-OAA and most ^{14}C-malate on a percentage basis.

Aspartate was not an important product except when pyruvate was added alone. The amount of ^{14}C incorporated into other compounds ranged from 15 to 35% and occurred largely

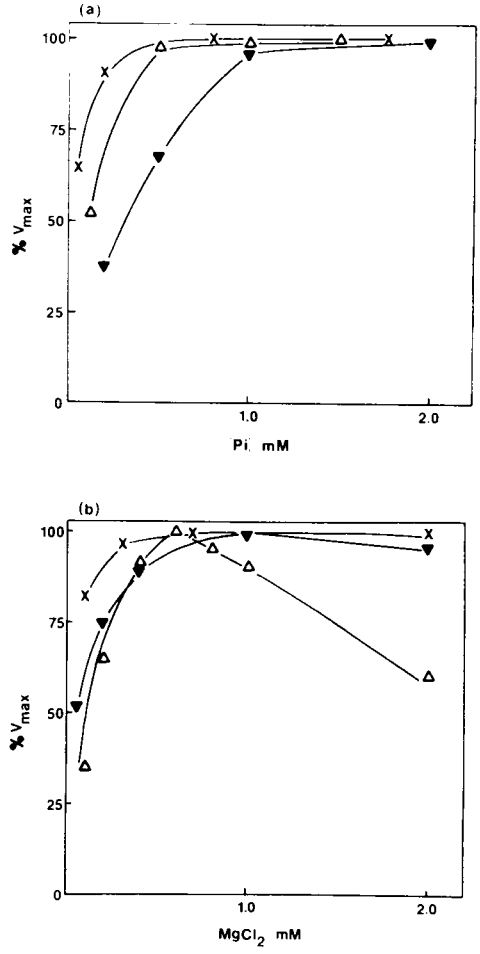

FIG. 3. Effect of the concentration of KH_2PO_4 (a) and $MgCl_2$ (b) on the rate of CO_2 fixation in the presence of pyruvate + OAA by mesophyll cells (△——△), mesophyll protoplasts (▼——▼), and protoplast extracts (×——×) of *Digitaria sanguinalis*. Maximum velocities attained at saturating salt concentrations of Pi and Mg were, respectively: mesophyll cells, 32 and 35; mesophyll protoplasts, 53 and 51; and protoplast extracts, 192 and 181 μmoles/mg Chl·hr.

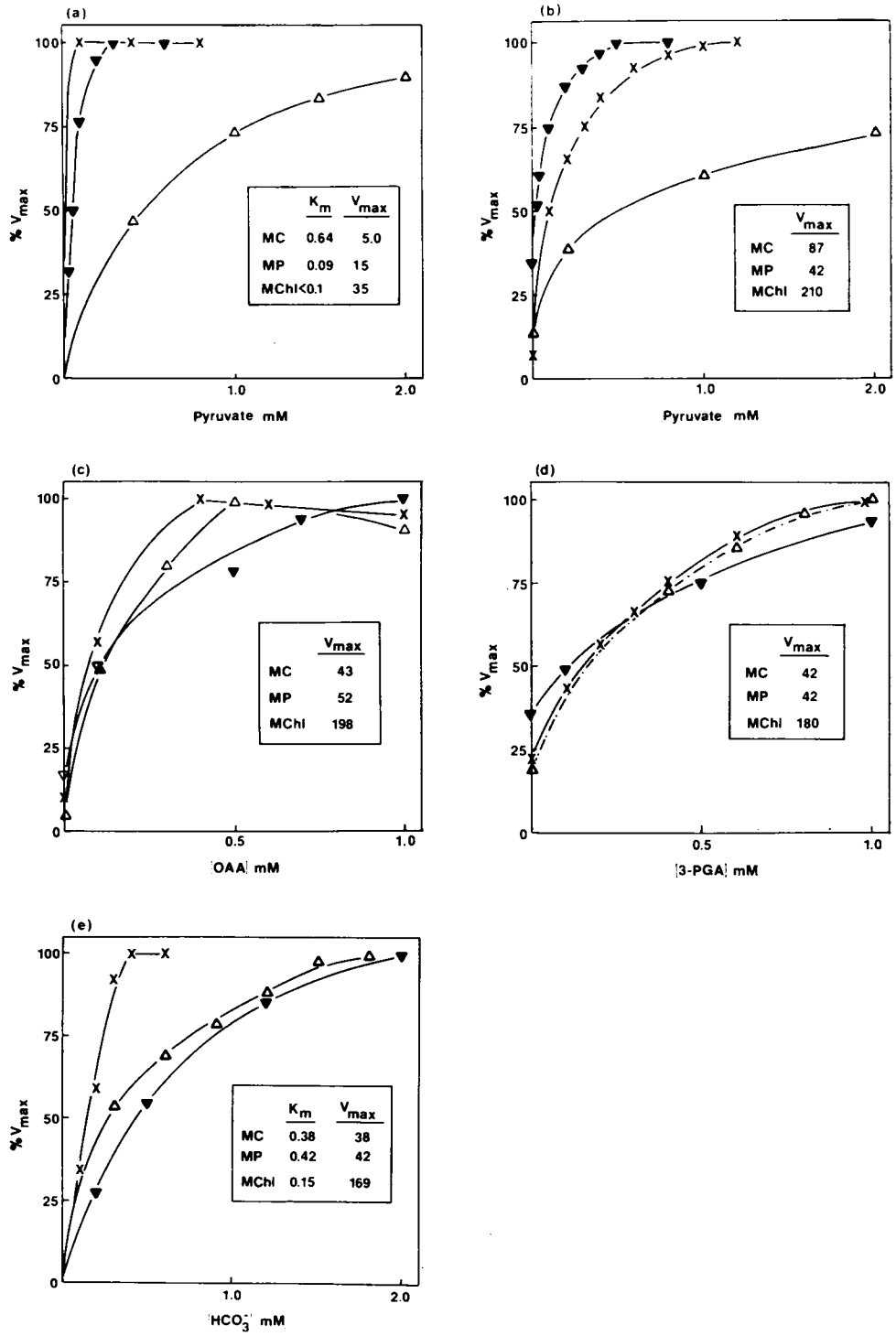

FIG. 4. Effect of concentration of substrates on CO_2 fixation by mesophyll cells, MC (△——△); mesophyll protoplasts, MP (▼——▼); and protoplast extracts, MChl (×——×) of *Digitaria sanguinalis*. Substrates varied were: pyruvate (a); pyruvate + 0.5 mM OAA (b); OAA + 5 mM pyruvate (c); PGA + 5 mM pyruvate (d); and HCO_3^- + 5 mM pyruvate + 0.5 mM OAA (e).

in one product which is relatively mobile in both dimensions but is as yet unidentified. No labeled products were detected as PGA or hexose phosphates.

Effect of DCMU on CO_2 Fixation. In an effort to understand the synergistic effect of the combination of pyruvate + OAA and pyruvate + PGA, the effect of DCMU on mesophyll protoplast CO_2 fixation was studied. DCMU, at a concentration of 1 μM, reduced the rate of pyruvate + OAA-induced CO_2 fixation to the rate obtained when pyruvate alone is added (Fig. 5a). When DCMU was added to a reaction mixture that contained pyruvate alone, the rate of CO_2 fixation was enhanced. The enhancement by DCMU of the pyruvate-induced CO_2 fixation was unexpected; however, this experiment has been repeated several times with similar results. Although there was some variation in the level of pyruvate induction with day to day experiments, the DCMU stimulation was consistent.

Table II. *Product Distribution of $^{14}CO_2$ Fixation by Mesophyll Protoplast Extracts of Digitaria sanguinalis*

Experiments were run in the light for 10 min. See "Materials and Methods" section for details of killing and chromatography procedures.

Addition	CO_2 Fixtion Rate	Total ^{14}C Fixed				Total in C_4 Acids
		Malate	Aspartate	OAA	Others	
	μmoles/mg Chl·hr	%				
5 mM pyruvate	40	28	40	17	15	85
5 mM pyruvate + 0.5 mM OAA	253	22	ND[1]	59	19	81
5 mM pyruvate + 0.5 mM PGA	221	46	1	38	16	84
5 mM pyruvate + 0.5 mM OAA + 1 mM PGA	285	35	ND	43	22	78
5 mM pyruvate + 0.5 mM OAA + 0.5 mM NH_4Cl	220	29	ND	43	28	72
5 mM pyruvate + 2 mM α-keto-glutarate + 0.5 mM NH_4Cl	37	31	1	32	36	64

[1] None detected.

The enhancement of the pyruvate-dependent CO_2 fixation by PGA was also found to be sensitive to DCMU (Fig. 5b).

Stoichiometry of CO_2 Fixation and O_2 Evolution. The stimulation of pyruvate-dependent CO_2 fixation by PGA and OAA in C_4 mesophyll preparations appears to be related to noncyclic electron transport, because the stimulation is DCMU sensitive. In order to test the relationship between light-dependent O_2 evolution and CO_2 fixation, these parameters were measured simultaneously with various substrates. In the presence of pyruvate alone there was no light-induced O_2 evolution with protoplast extracts, but rather a low rate of light-induced O_2 consumption (5 μmoles/mg Chl·hr), although there was light-induced fixation of CO_2 (19.6 μmoles/mg Chl·hr). With OAA added alone at 0.5 mM there was also no detectable light-dependent O_2 evolution (CO_2 fixation: 7.8 μmoles/mg Chl·hr), although addition of pyruvate following the OAA induced high rates of O_2 evolution. Therefore, both OAA and pyruvate were needed in combination to induce photosynthetic O_2 evolution as well as maximum rates of CO_2 fixation.

With addition of pyruvate + OAA to protoplast extracts the ratio of O_2 evolved to CO_2 fixed was about 0.5/1 after an initial lag period of CO_2 fixation (Fig. 6). With pyruvate + PGA as substrates the O_2 evolution/CO_2 fixation ratios at time intervals of 2.5, 5.0, 7.5, and 10 min after initiation of the reaction were 0.76, 0.77, 0.74, and 0.78, respectively.

DISCUSSION

Although mesophyll cells of C_4 plants lack the carboxylative phase of the Calvin pathway, the presence of certain enzymes has been taken to indicate that C_4 mesophyll cells catalyze a number of photosynthetic reactions. Pyruvate Pi dikinase, PEP carboxylase, and NADP-malic dehydrogenase of the C_4 mesophyll cells would function in sequence to catalyze the conversion of pyruvate and CO_2 to malate utilizing 1 NADPH and 2 ATP (8, 13, 25). Although the enzyme potential is present in mesophyll cells for such conversions, in previous studies pyruvate-induced CO_2 fixation in mesophyll cells was about $\frac{1}{20}$ of the capacity expected on the basis of enzyme potential and whole leaf photosynthetic capacity (6, 10). While PEP induces very high rates of CO_2 fixation with C_4 mesophyll cells and protoplasts (5, 6, 8, 10, 12, 33) this occurs both in the light and the dark. Using PEP as a substrate eliminates the requirement for ATP in the conversion of pyruvate to PEP, so the carboxylation capacity does not measure the chloroplast ability for making ATP or NADPH, for the products of the PEP induction are largely OAA (5, 33).

The results of the present study with *Digitaria sanguinalis* indicate that C_4 mesophyll chloroplasts are functional for making both ATP and NADPH and that the maximum conversion of pyruvate to PEP (which would require ATP), carboxylation to OAA, and subsequent reduction to malate (using NADPH) occurs when there is a parallel requirement for both ATP and NADPH which allows noncyclic electron flow to operate at its maximum potential. The requirement for both ATP and NADPH is induced by the presence of pyruvate + OAA or pyruvate + PGA.

Previous studies on intercellular enzyme distribution indicate that both C_4 mesophyll cells and bundle sheath cells have 3

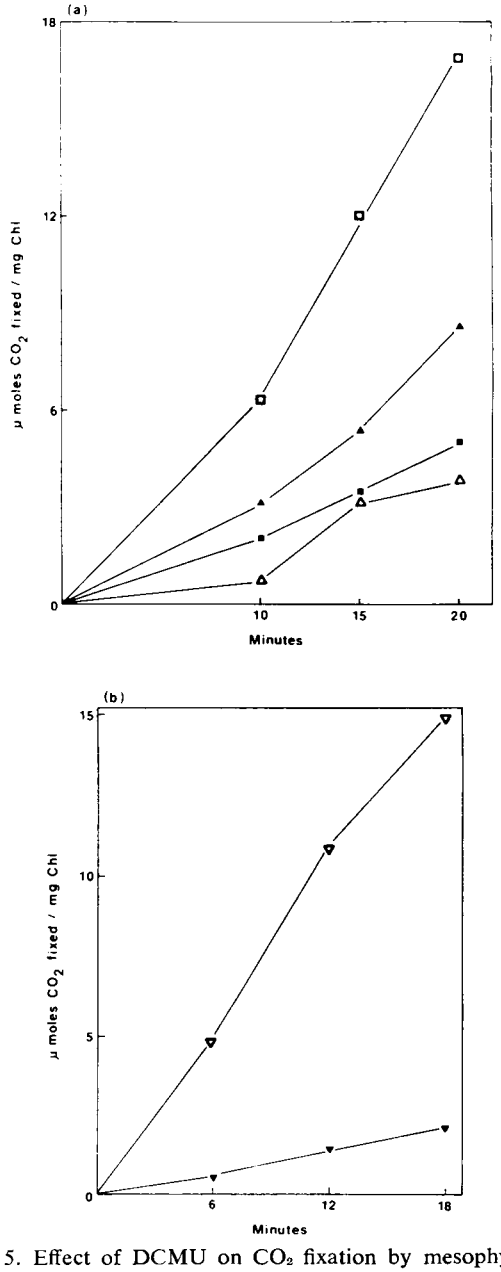

FIG. 5. Effect of DCMU on CO_2 fixation by mesophyll protoplasts of *Digitaria sanguinalis* induced by pyruvate ± OAA (a) and pyruvate + PGA (b). Conditions tested were pyruvate (△——△); pyruvate + OAA (□——□); pyruvate + PGA (▽——▽); pyruvate + 1 μM DCMU (▲——▲); pyruvate + OAA + 1 μM DCMU (■——■); pyruvate + PGA + 1 μM DCMU (▼——▼).

P-glycerate kinase and NADP triose P-dehydrogenase, enzymes for reduction of PGA to triose phosphate (5, 9, 15, 25, 34), and phosphoglyceromutase and enolase, enzymes for the conversion of PGA to PEP (5, 23).

The induction of CO_2 fixation in the C_4 mesophyll preparations by various substrates is discussed with respect to enzyme complement, energy requirements, and photochemical capacity.

Characteristics of Mesophyll CO_2 Fixation. The mesophyll preparations of *D. sanguinalis* did not fix CO_2 at appreciable rates without added substrates nor with the addition of ribose-5-P (Table I), which is consistent with intercellular enzymes studies suggesting a lack of an independent CO_2 fixation cycle and lack of RuDP carboxylase in mesophyll cells (4, 5, 10, 12, 25). Pyruvate is proposed as the precursor for the C_4 pathway in mesophyll cells, but the induction of CO_2 fixation by pyruvate with the three mesophyll preparations was relatively low (Table I). However, the combinations of pyruvate + OAA and pyruvate + PGA were found to increase 4 to 6-fold the rates obtained with pyruvate alone. Low rates of CO_2 fixation were induced when OAA and PGA were added separately. OAA in the absence of pyruvate presumably induced some CO_2 fixation by the generation of pyruvate via a spontaneous decarboxylation. The induction of CO_2 fixation by PGA in the absence of pyruvate may be largely due to conversion of 3-PGA to 2-PGA to PEP by phosphoglyceromutase and enolase, respectively, which may occur in the dark or light. The rates induced by PGA alone are comparable to the level of enolase and phosphoglyceromutase reported for crabgrass mesophyll cells (Ku and Edwards, unpublished).

Whereas maximum rates were obtained with a combination of pyruvate + PGA + OAA with the three types of mesophyll isolations, in absolute terms, the rates obtained with protoplast extracts were always highest. This result suggests that some rate-limiting step of substrate uptake is occurring at the plasma membrane. Typically, maximum rates of 250 to 350 μmoles/mg Chl·hr were obtained with protoplast extracts; these rates are clearly high enough to be of significance in whole leaf photosynthesis.

The pyruvate + OAA induction of CO_2 fixation with once pelleted chloroplasts was about 20% of the CO_2 fixation obtained with the complete protoplast extract. Chloroplasts washed once had only 2% of the activity of the initial protoplast extract, and addition of the original supernatant to the chloroplasts largely restored the capacity for CO_2 fixation. Although only a small percentage of the PEP carboxylase is associated with once pelleted chloroplasts (<5% of total activity), this is enough to sustain a rate of CO_2 fixation of approximately 100 μmoles/mg Chl·hr. Despite the evidence for extrachloroplastic PEP carboxylase in our preparations (11), some researchers have shown low rates of light-dependent PEP-induced CO_2 fixation with chloroplast preparations, pelleted once, from C_4 plants (3, 37, 38). The meaning of the light requirement with the PEP induction is unknown although the low percentage of PEP carboxylase associated with chloroplasts after a single centrifugation is readily lost if the chloroplasts are washed (28).

Composition of Reaction Mixture. The composition of the reaction mixture was found to be important but relatively simple for obtaining high rates of light-dependent CO_2 fixation. Inorganic phosphate and magnesium were required. Avron and Gibbs (2) showed that free magnesium ions inhibited photosynthetic CO_2 fixation in spinach chloroplasts, and that magnesium and EDTA could be eliminated without loss in activity. In the present studies, low levels of magnesium stimulated the pyruvate induction of CO_2 fixation. In this case

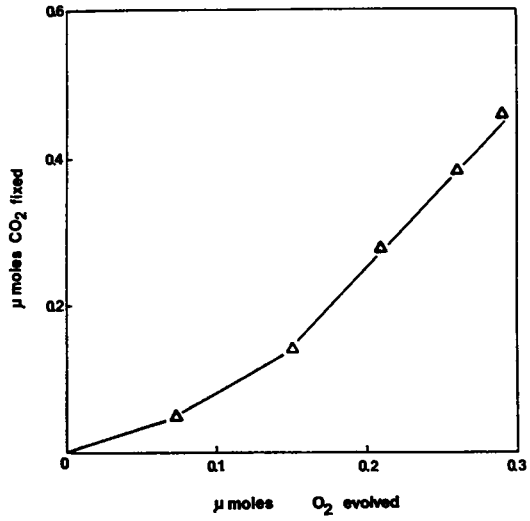

FIG. 6. Stoichiometry of CO_2 fixation and O_2 evolution by mesophyll protoplast extracts of *Digitaria sanguinalis* in the presence of 5mM pyruvate + 0.5 mM OAA. $^{14}CO_2$ fixation indicated the reaction velocity to be 94 μmoles/mg Chl·hr.

the magnesium stimulation may be due to the magnesium requirement of the cytoplasmic PEP carboxylase (35) or of other enzymes of the C_4 pathway. However, further studies are needed to determine the role of magnesium and also possible interactions between EDTA, magnesium, and manganese.

Mesophyll protoplasts and protoplast extracts showed rather broad pH optimums of CO_2 fixation centered at pH 7.5 with pyruvate + OAA, whereas mesophyll cells—for reasons unknown—have a double peak of activity which resembles the pH dependence of OAA-induced O_2 evolution with crabgrass mesophyll cells (33).

The induction of CO_2 fixation with all three mesophyll preparations in the absence of exogenous ADP and NADP is the best indication that endogenous nucleotides are retained (and are turning over) and that membrane integrity has not been lost.

Characteristics of Substrate Saturation. Because pyruvate Pi dikinase and NADP-malate dehydrogenase are chloroplastic enzymes (11, 15, 20), CO_2 fixation would require pyruvate uptake through the plasma membrane with mesophyll cells and protoplasts, and pyruvate uptake into the chloroplast with mesophyll cells, protoplasts, and protoplast extracts. Therefore, any determinations of the substrate concentrations required to give half-maximum reaction velocities represents both the Km of the enzyme for the substrate and any permeability barriers encountered. In the fixation of CO_2, HCO_3^- would not need to be taken up by the chloroplasts in our preparations, because PEP carboxylase was extrachloroplastic. The observation that chloroplast preparations saturated at lower levels of HCO_3^- than mesophyll cells and mesophyll protoplasts (Fig. 4e) suggests that bicarbonate encounters some barrier at the plasma membrane. The apparent Km's for HCO_3^- with crabgrass mesophyll cells, protoplasts, and protoplast extracts of 0.38, 0.42, and 0.15 mM, respectively, are similar to or lower than the concentration of HCO_3^- in solution under atmospheric conditions (calculated value about 0.3 mM at pH 7.5, 0.46 mM at pH 8, see ref. 36), which suggests carboxylation conditions in C_4 mesophyll cells are favorable for fixing atmospheric CO_2. In other studies, the chloroplast preparations saturated at lower levels of Pi, Mg^{2+}, and OAA than mesophyll cells or protoplasts, which again suggests that the plasma membrane may

represent a permeability barrier to these compounds. Mesophyll protoplasts and cells showed different characteristics when pyruvate was varied, plus or minus OAA, in which case, for unknown reasons, the concentration of pyruvate to give half-maximum velocity was about 6-fold higher with mesophyll cells. Whether this difference is a ramification of the presence of a cell wall or some effect of the different isolation procedures remains unclear.

The results with OAA and PGA as substrates indicate that *in vivo* levels would need to be around 0.4 mM and 1 mM, respectively, to give maximum stimulation of CO_2 fixation in mesophyll cells. Because both substrates may occur together *in vivo*, the concentration requirements for maximum induction of CO_2 fixation may be even lower than those indicated by our *in vitro* experiments. Hatch (14) has suggested the *in vivo* concentration of PGA in photosynthetic tissue of maize during steady state photosynthesis may be at least 6 mM, and evidence with nonaqueous chloroplast separations suggests a major part of the PGA may be in mesophyll chloroplasts (34). Although OAA appears as an initial product of CO_2 fixation in C_4 plants along with malate and aspartate (16, 17), neither the pool size nor concentration in the leaf has been determined.

Because the addition of OAA is required to give maximum rates of pyruvate induction of CO_2 fixation, it is apparent that the pyruvate induction alone is not generating a high enough concentration of OAA *in vitro* to support maximum induction or to be self-sustaining. This result was true with mesophyll cells, protoplasts, and protoplast extracts inasmuch as exogenous OAA was stimulatory (Table I, Fig. 4c). An example indicating the levels of OAA formed during pyruvate induction can be given. In one experiment there was a maximum rate of pyruvate induction of 40 μmoles/mg Chl·hr with a protoplast extract preparation in which 17% of the products were oxaloacetate after 10 min (Table II). The concentration of OAA was calculated to be less than 0.01 mM after 10 min of fixation (Chl level 2 μg/250 μl reaction mixture). This concentration is well below the level of OAA needed for significant induction of CO_2 fixation by this substrate (Fig. 4c). *In vivo* the OAA concentration in mesophyll cells may be higher than *in vitro*, because there is not a large volume of medium external to the chloroplasts or cells allowing dilution of the OAA produced as may occur *in vitro*.

Products of CO_2 Fixation. Consistent with the proposed function of the C_4 pathway in mesophyll cells of C_4 plants is our finding that C_4 acids are the primary products of CO_2 fixation with protoplast extracts (Table II). Malate, the proposed transport metabolite from mesophyll to bundle sheath cells, accounted for 22 to 46% of the total CO_2 fixed. The percentage of the total incorporated radioactivity found in malate was lowest when exogenous OAA was added; this condition is predictable, because the exogenous OAA would dilute the ^{14}C-OAA produced by CO_2 fixation and compete with the ^{14}C-OAA for reduction to malate. Malate was a significant product (46%) when a combination of pyruvate and PGA was added to the chloroplast reaction mixture. This result suggests that the pyruvate + PGA induction also involves significant OAA reduction to malate.

In studies with whole leaf photosynthesis, it has been determined that *D. sanguinalis*, as with most C_4 plants, forms both malate and aspartate as early photosynthetic products (23). In our studies with protoplast extracts, appreciable amounts of aspartate were detected only when pyruvate was added alone, a condition in which the level of OAA generated may be too low to rapidly utilize the NADPH for reduction to malate. In NADP-malic enzyme species such as crabgrass, maize, and sugarcane, the nature of the rapid aspartate formation is unclear, although these species have substantial levels of aspartate aminotransferase in mesophyll cells (11, 20, 23, Gutierrez and Edwards, unpublished). To determine if aspartate formation could be induced by NH_4^+, combinations of pyruvate + OAA + NH_4^+ and pyruvate + α-ketoglutarate + NH_4^+ were tested. The addition of NH_4^+ did not influence the rate of CO_2 fixation or induce the synthesis of aspartate.

DCMU Inhibition of CO_2 Fixation. Because the pyruvate induction of CO_2 fixation is thought to require ATP for pyruvate Pi dikinase, it is of interest to consider whether the ATP is generated by cyclic or noncyclic photophosphorylation with various substrates. The pyruvate induction of light-dependent CO_2 fixation was not inhibited by DCMU, an inhibitor of noncyclic electron flow. Rather, 1 μM DCMU caused some stimulation of the pyruvate-induced CO_2 fixation. Although unexpected, the stimulation of the pyruvate induction by DCMU is compatible with previous evidence for a DCMU stimulation of cyclic photophosphorylation in spinach chloroplasts (1). The lack of inhibition by DCMU suggests that the ATP for the pyruvate induction is generated by cyclic photophosphorylation. DCMU did inhibit the OAA and PGA enhancement of the pyruvate-dependent CO_2 fixation, which suggests that OAA and PGA stimulate CO_2 fixation by inducing ATP synthesis through coupled noncyclic electron flow (Fig. 5). The results suggest that when noncyclic electron flow is low (either low levels of OAA and PGA or inhibition by DCMU), cyclic photophosphorylation is the major source of ATP. However, from the present experiments, we cannot estimate the contribution of cyclic photophosphorylation under conditions for optimum noncyclic electron transport (*i.e.* saturating OAA or PGA).

Stoichiometry of CO_2 Fixation and O_2 Evolution. Hatch and Kagawa (15), Salin *et al.* (33), and Salin and Black (32) have shown that OAA and PGA induce light-dependent O_2 evolution in C_4 mesophyll preparations, which suggests an NADPH requirement in C_4 mesophyll cells both for OAA reduction and PGA reduction. Contrary to our findings, Salin and Black (32) found the PGA induction of O_2 evolution was not associated with CO_2 fixation. Kagawa and Hatch (22) have shown with isolated mesophyll chloroplasts of *Atriplex spongiosa* that pyruvate stimulated the O_2 evolution induced by OAA or PGA, which was interpreted as a stimulation of noncyclic electron flow due to the ATP requirement by pyruvate Pi dikinase.

By measuring O_2 evolution and CO_2 fixation simultaneously by mesophyll protoplast extracts with various substrates, the relationship between CO_2 fixation and substrate reduction was tested. With pyruvate there was no induction of O_2 evolution with mesophyll protoplast extracts of *D. sanguinalis*, although some CO_2 fixation occurred. This result suggests that under these conditions cyclic photophosphorylation provides the ATP for pyruvate conversion to PEP, a conclusion supported by lack of DCMU inhibition of the pyruvate induction (Fig. 7a). OAA, at 0.5 mM, did not induce photosynthetic O_2 evolution in mesophyll preparations, whereas 0.5 mM OAA in combination with 5 mM pyruvate induced high rates of O_2 evolution paralleled by CO_2 fixation with an O_2/CO_2 ratio approximating 0.5 (Fig. 6). The failure of OAA alone to induce the Hill reaction in our studies does not agree with the results of Hatch and Kagawa (15) and Salin *et al.* (33), in which OAA was reported to induce photosynthetic O_2 evolution with C_4 mesophyll preparations. We interpret this discrepancy as indicating that in their experiments either OAA generated enough pyruvate due to OAA lability to give some pyruvate + OAA induction, or the OAA induction occurred through uncoupled noncyclic electron flow in which there was no synthesis of ATP, or both.

The ratio of O_2 evolved/CO_2 fixed with pyruvate + OAA

approximated 0.5 and agrees well with a proposed stoichiometry of noncyclic electron flow for ATP and NADPH synthesis of 2 ATP and 1 NADPH for 0.5 O_2 evolved (18, 31, 39), and the energy requirements for conversion of pyruvate + CO_2 to malate. This relationship is illustrated in Figure 7b.

Pyruvate + PGA also induced both light-dependent O_2 evolution and CO_2 fixation with a stoichiometry of about 0.75 μmole O_2 evolved/μmole CO_2 fixed. Assuming 1 NADPH and 2 ATP generated per 0.5 O_2 evolved, and considering that PGA reduction to triose phosphate would require 1 ATP and 1 NADPH, a net of 1 ATP would be generated per 0.5 O_2 evolved, which could be used for pyruvate conversion to PEP. The theoretical stoichiometry for O_2 evolution to CO_2 fixation in the pyruvate + PGA induction system is shown in Figure 7c, in which case the ratio of O_2 evolved/CO_2 fixed would be 1. We observed a lower ratio, about 0.75, with the pyruvate + PGA induction and also found that about half of the CO_2-fixation products with pyruvate + PGA were malate (Table II). Therefore, in the pyruvate + PGA induction of CO_2 fixation, a combination of the reactions shown in Figure 7, b and c, may be occurring due to the rapid formation of OAA. This complexity, in addition to some dark induction by PGA, may tend to lower the O_2/CO_2 ratio below the theoretical value of 1. Additional experiments are needed with various combinations of substrates in which labeled OAA and PGA are used and the rate of product reduction evaluated with respect to the rate of O_2 evolution and CO_2 fixation.

In considering the energy requirements for CO_2 fixation in *D. sanguinalis*, an NADP-malic enzyme species, 5 ATP, and 2 NADPH are suggested per CO_2 fixed (2 ATP for the C_4 cycle and 3 ATP, 2 NADPH for the Calvin cycle) (8, 29). The requirements for 2 NADPH/CO_2 fixed would correspond with 1 O_2 evolved giving the expected O_2/CO_2 of 1. This requirement is based on the assumption that all carbon flow is through the Calvin pathway and for each CO_2 fixed by RuDP carboxylase in bundle sheath cells the resulting products, 2(3-PGA), would require 2 NADPH for reduction. Half of the requirement for reductive power would come from mesophyll cells in the form of malate, which would generate equivalent amounts of CO_2 and NADPH through NADP-malic enzyme in the bundle sheath cells. Due to relatively low levels of photosystem II in bundle sheath cells of NADP-malic enzyme species, part of the PGA formed in bundle sheath cells may be transported to mesophyll cells for reduction (29) in which case the PGA induction of noncyclic electron flow could provide additional ATP for pyruvate conversion to PEP. This additional generation of ATP would seem in excess of that needed, because the energy proposed from noncyclic electron transport would satisfy the requirements for conversion of pyruvate and CO_2 to malate (Fig. 7b). However, in a somewhat similar manner a stoichiometry of 4 ATP to 2 NADPH per 1 O_2 evolved in C_3 plants would also provide excess ATP, considering the requirement of 3 ATP and 2 NADPH per CO_2 fixed in the Calvin pathway. The results with C_4 mesophyll preparations suggest that in the presence of pyruvate + PGA + OAA the potential for pyruvate conversion to PEP may be higher than the potential for OAA conversion to malate due to the additional ATP generated by PGA-dependent noncyclic photophosphorylation. Additional ATP generated by cyclic photophosphorylation and PGA-dependent noncyclic photophosphorylation may tend to keep the pyruvate concentration low, maintaining a strong concentration gradient for diffusion of pyruvate from bundle sheath to mesophyll cells. Another factor to consider is that aspartate is also an early product of photosynthesis in C_4 plants although *in vitro* we have not obtained high levels of aspartate as a product in crabgrass mesophyll preparations.

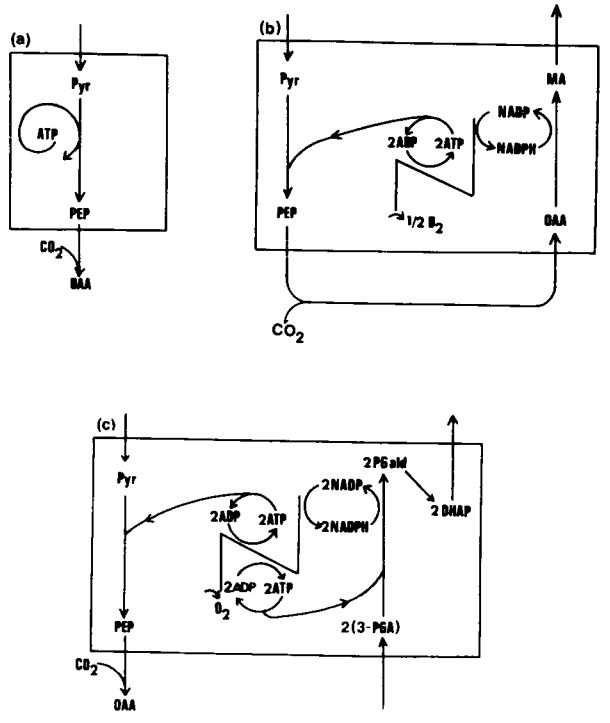

FIG. 7. Schemes illustrating proposed energetics of pyruvate-dependent inductions of CO_2 fixation in C_4 mesophyll cells in relation to cyclic and noncyclic electron flow. (a) pyruvate, (b) pyruvate + OAA, (c) pyruvate + PGA.

Consistent with the often proposed role of the mesophyll cells in C_4 photosynthesis is our demonstration of high rates of pyruvate-dependent CO_2 fixation in isolated mesophyll cells, protoplasts, and protoplast extracts. Our results suggest that for *D. sanguinalis*, regulation of carboxylation in mesophyll cells may be mediated by the levels of OAA and PGA available for reduction.

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