LIGHT/PHOSPHOENOLPYRUVATE DEPENDENT CARBON DIOXIDE FIXATION BY ISOLATED SUGAR CANE CHLOROPLASTS

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The major process of photosynthetic carbon dioxide fixation in algae and temperate plants is the carbon reduction cycle of Bassham and Calvin (1957), although β-carboxylation reactions may also account for a significant amount of the total carbon fixed in the light (Walker, 1962). Recently it has been suggested on the basis of short term $^{14}CO_2$ labelling patterns (Kortschak, Hartt and Burr, 1965; Hatch and Slack, 1966) and enzyme activities (Slack and Hatch, 1967), that in sugar cane and related tropical grasses β-carboxylation reactions may be of even greater importance. Although it is generally accepted that photosynthesis activity may increase rates of β-carboxylation by supplying the three carbon acceptor, phosphoenolpyruvate (PEP), so far no direct connection between rates of β-carboxylation and electron transport has been demonstrated. However, we now report a light-stimulated carboxylation of PEP by isolated sugar cane chloroplasts, and suggest that light may stimulate the reaction by photoreduction of the initial product oxalacetate (OAA), to malate.

Chloroplasts were prepared by grinding 10 g. mature cane leaves in 200 ml. pyrophosphate media (Cockburn, Walker and Baldry, 1968), as previously (Baldry, Coombs and Gross, 1968) but with 0.002 M $MgCl_2$, 0.002 M $MnCl_2$, 0.004 M versene, 0.005 M iso-ascorbate, 0.001 M thioglycollate and 1% polyethylene glycoll 4000. The additional components were necessary to reduce inhibition by endogenous phenols released during grinding of the cane (Gross, Baldry, Bucke and Coombs, 1969). CO_2 fixation was measured as described by Bucke, Walker and Baldry (1966). Tubes containing chloroplasts (approximately 100 μg chlorophyll), cofactors as required, and 1 μ mole of $NaH^{14}CO_3$ of known specific activity in 300 μl of media (0.33 M sorbitol, 0.05 M N-2-hydroxymethylpiperazine-N'-2-ethanesulphonic acid, and 0.001 M $MgCl_2$, pH 7.0) were suspended in a constant temperature water bath (25°) illuminated by a quartz iodine lamp (60,000 lux). Dark tubes were wrapped in aluminium foil. 10 μl samples were taken at time intervals.

As shown in table 1, no significant CO_2 fixation was observed in the absence of added substrate or with ribose-5-phosphate, indicating that the photosynthetic carbon reduction cycle was inactive in these preparations. However, in the light with PEP as substrate, $^{14}CO_2$ incorporation was linear over periods of time up to 45 minutes, with

Table 1. Substrate specificity for CO_2 fixation by isolated cane chloroplasts, rates are μ moles CO_2 fixed/mg. chlorophyll/hr.

Conditions	Rate	Conditions	Rate
light, no additions	trace	light, pyruvate (2)	0
light, ribose-5-P	trace	light, pyruvate (2), ADP (2)	0
dark, PEP (2)	0.1	light, pyruvate (2), ATP (2)	trace
light, PEP (2)	1.7	light, PEP (10)	2.5

Table 2. Effects of additives on PEP (2 μ moles) dependent CO_2 fixation by isolated cane chloroplasts. Results for treated samples are expressed as a percentage of a parallel control sample run in the light.

a. Glutamate-oxalacetate transaminase

dark, transaminase	10%	light, transaminase	100%
dark, transaminase, glutamate (2)	30%	light, transaminase, glut.	30%

b. Inhibitors (all reactions in light)

DCMU (10^{-4})	10%	o-phenanthroline (10^{-3})	25%
isoniazide (10^{-3})	100%	Iodoacetamide (10^{-3})	46%
malonate (10^{-3})	54%	PCMB (10^{-4})	4%
Versene (10^{-2})	0%	inorganic phosphate (10^{-1})	10%

c. Nicotinamide adenine dinucleotide phosphate (NADP), all samples included added spinach ferredoxin and ferredoxin/NADP reductase). All reactions in light.

NADP μ moles	0	0.1	0.5	1.0	2.0
% of control	120	100	90	50	10

d. Phosphoglyceric acid and oxalacetate

PGA (2)	50%
OAA (2)	0%

Figures in parentheses represent μ moles or molar concentration of additives

maximum rates in the order of 2 μ moles CO_2 fixed per mg. chlorophyll per hour. The products of the reaction were investigated by chromatography and autoradiography as described by Benson et al (1950). Amino acids were detected using ninhydrin and organic acids using bromocresol purple. Ketoacids were trapped as their dinitrophenyl-hydrazones, separated by chromatography and detected by autoradiography as described by Coombs and Volcani (1968). Almost all the radioactivity was located in oxalacetate (10%) and malate (90%). There was little evidence that oxalacetate was being further metabolised by endogenous transaminase to aspartate.

PEP could not be replaced by pyruvate plus ADP or ATP, (table 1). Addition of commercial glutamate-oxalacetate transaminase (Sigma, London) did not affect the rate of CO_2 fixation in the light (table 2a). However, the further addition of glutamate as amino donor stimulated dark CO_2 fixation, but inhibited the reaction in the light.

DCMU (3-(3-4-dichlorophenyl)-1-1-dimethylurea) and o-phenanthroline, inhibitors of photosynthetic electron transport, markedly reduced the fixation of CO_2, whereas isoniazide, an inhibitor of transaminase, had little effect (table 2b). The actions of other inhibitors were similar to those summarised by Walker (1962) for inhibition of PEP carboxylase.

These results are consistent with fixation of CO_2 by PEP carboxylase with subsequent photoreduction to malate, possibly mediated via photosynthetically reduced nicotinamide adenine dinucleotide phosphate (NADPH). However, as shown in table 2c addition of spinach ferredoxin, ferredoxin/NADP reductase and NADP resulted in an inhibition of CO_2 fixation. Lineweaver-Burk analysis of kinetics of inhibition indicated (Figure 1) that NADP was a competitive inhibitor of the light/PEP dependent CO_2 fixation by isolated cane chloroplasts.

Addition of phosphoglyceric acid, (table 2d), also inhibited the reaction, as did OAA. The inhibition by oxalacetate suggests that light stimulates CO_2 fixation by rapid photoreduction of the oxalacetate produced by PEP carboxylase preventing end product inhibition of the carboxylation reaction.

After grinding cane leaves in pyrophosphate/sorbitol media and filtering through muslin the resultant brei was fractionated by differential centrifugation and the PEP carboxylase activity determined, in the dark with added glutamate and OAA/glutamate transaminase. Although much of the activity was recovered from the final supernatant (table 3) the highest specific activity was observed in a fraction precipitated in 45 minutes at 140,000 x g. After vigorous ultrasonic disruption of the chloroplast fraction for one minute about 20% of the activity could be precipitated

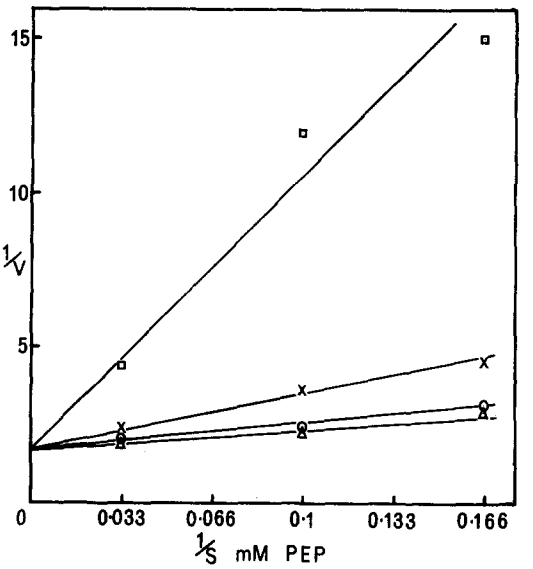

Figure 1. Lineweaver-Burk plot showing competitive inhibition by NADP of Light/PEP dependent CO_2 fixation by isolated sugar cane chloroplasts; (□), 2 μ moles NADP; (X), 1 μ mole NADP; (O), 0.5 μ mole NADP; (Δ), No NADP.

Table 3. Distribution of PEP carboxylase in various fractions derived from sugar cane leaves by differential centrifugation (reaction mixture contained 1 μ mole PEP, 10 μ moles $MgCl_2$, 10 μ moles glutamate, 1 μl transaminase, 1 μ mole $NaH^{14}CO_3$, 180 μ moles TRIS, pH 8.3. The major product was aspartate).

Fraction, g, minutes	Relative specific activity	Mg. Protein
Chloroplasts, 4,000 xg, 1 min.	100	22
25,000 x g, 20 min.	150	12
140,000 x g, 45 min.	1,425	2
Supernatant	550	41

by similar centrifugation. This suggests that the high specific activity fraction could represent chloroplast membrane fragments or particles liberated from broken chloroplasts during grinding of the leaf.

Conclusions. Our results are consistent with the fixation of CO_2 by a chloroplast membrane-bound PEP carboxylase followed by a rapid photoreduction, possibly mediated by ferredoxin, of the oxalacetate to malate. These results would account for the rapid

labelling of malate observed in vivo when sugar cane leaves are exposed to $^{14}CO_2$ in the light (Kortschak, Hartt and Burr, 1965; Hatch and Slack, 1966). Since the rates of CO_2 fixation by our chloroplasts are relatively low the exact significance of β-carboxylation in relation to total photosynthetic CO_2 fixation is hard to assess. However, if all the PEP carboxylase activity recovered in the supernatant after grinding cane leaves by our technique had its origin in the chloroplasts, β-carboxylation could play a major part in accounting for the high rates of photosynthesis and the ability of sugar cane to reduce CO_2 concentration of the atmosphere to near zero (Photosynthesis in Sugarcane, 1968).

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