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RuP_2 pool size indicated by CO_2 assimilation following the abrupt loss of light

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Abstract. Measurement of the changes in CO_2 uptake by single leaves following the abrupt onset of darkness were made on sugarbeets (*Beta vulgaris* L.) and (*Phaseolus vulgaris* L.) The shape of the CO_2 dark response curve was analyzed with respect to the reaction kinetics of CO_2, RuP_2 and RuP_2 carboxylase. It was concluded that the net uptake of CO_2 in the dark from a 1% O_2 atmosphere can be approximately related to the pool size of the RuP_2 substrate in the chloroplasts of C_3 plants. This information was combined with CO_2 levels and decay rates of the response curves to infer changes in carboxylase activity. Preliminary data are presented showing the relative concentration changes in RuP_2 as light intensity decreases and as water stress increases. The method may prove useful in studies of plant response to environmental stresses.

Introduction

The amount of ribulose-1,5-biphosphate (RuP_2) in leaves is of increasing practical interest because there may be times when it limits CO_2 assimilation rates [9]. The generation of RuP_2 requires energy developed by light driven electron transfer. In C_3 plants where the CO_2 fixed by PEP carboxylase is small, and when O_2 levels are purposely kept low, RuP_2 reacts nearly stoichometrically with CO_2. Under these conditions, measurements of net CO_2 assimilation immediately following the onset of darkness may be useful in estimating the pool size of RuP_2 in the chloroplasts. The work reported here was undertaken to explore this possibility and to consider how it might be used experimentally.

Methods

Transpiration and CO_2 uptake were measured by standard methods as previously described [1], except that the gas exchange chamber was modified to accomodate intact leaves on potted plants. This was accomplished by cutting a slit in the side of the outermost chamber so that the leaf could be passed through it and into the assimilation cell. The slit was sealed around the

leaf or its petiole with noncorosive silicon rubber*. The volume of the gas exchange cell and the gas lines to the analyzer was reduced as much as possible to decrease the lag time between instrument response and changes in CO_2 assimilation. Specifically, the area of leaf tissue in the cell was 19.6 cm^2, the volume of the gas in the cell surrounding this part of the leaf was 20 ml and the volume of gas in the connecting lines was about 15 ml. The flow rate was 1 l min^{-1} and the boundary was 110 s m^{-1}. Leaf temperature was 19± 0.5 °C. An incandescent flood lamp was used with a filter that was submerged in the water bath. The filter blocked most light outside of the photosynthetically active range. The composition of gas flowing over the leaf was always adjusted to 1% O_2.

The plants in the greenhouse were grown in pots filled with a sand, vermiculite and peat mix containing adequate plant nutrients. Supplemental lighting maintained at least 1200 $\mu E\ m^{-2}\ s^{-1}$. The beet leaves were studied when the plants were about 30 cm tall with 15 to 20 leaves. The bean leaves were on plants sufficiently mature to produce blossoms and runners. The leaves chosen for the measurements were near the end of their rapid expansion growth phase.

The CO_2 dark response curves (Figure 1) were traced on chart paper running 0.4 mm s^{-1} through a recorder connected to a CO_2 gas analyzer. The total change in the gas stream CO_2 levels from light to dark is expressed as a leaf fixation rate in Figures 1 and 2. They are based on the absolute amount of CO_2 in the gas stream leaving the leaf chamber. Thus the fixation rates include the dark respiration of CO_2, but not any CO_2 fixed by PEP carboxylase if the fixation by PEP carboxylase remained constant during each observation. The areas under the curves were measured with a planimeter interfaced with a small computer programmed to give net CO_2 assimilation during the time period in which the curve was traced. These values for areas were corrected for lag time of the instrument. The magnitude of this correction depends on the change in gas concentration associated with the change from maximum to minimum CO_2 uptake rates, the volume of gas in the leaf container, the volume of gas in the connecting lines and in the CO_2 analyzer chamber, and the flow rate. A family of curves like those shown in Figure 1 were produced by using aluminium foil in place of a leaf and abruptly increasing the concentration of CO_2 flowing into the chamber. The areas under this family of curves were plotted as a function of their intercepts on the vertical axis. For our system this produced a nearly straight calibration curve with an intercept on the horizontal axis at 7 mg CO_2 $m^{-2}\ s^{-1}$. It was used to correct the areas under leaf curves for instrument lag time.

*Dow Corning 738 RTV (noncorrosive electronics grade). Trade names and company names are included for the benefit of the reader and do not imply any endorsement or prefertential treatment of the product listed by the US Department of Agriculture.

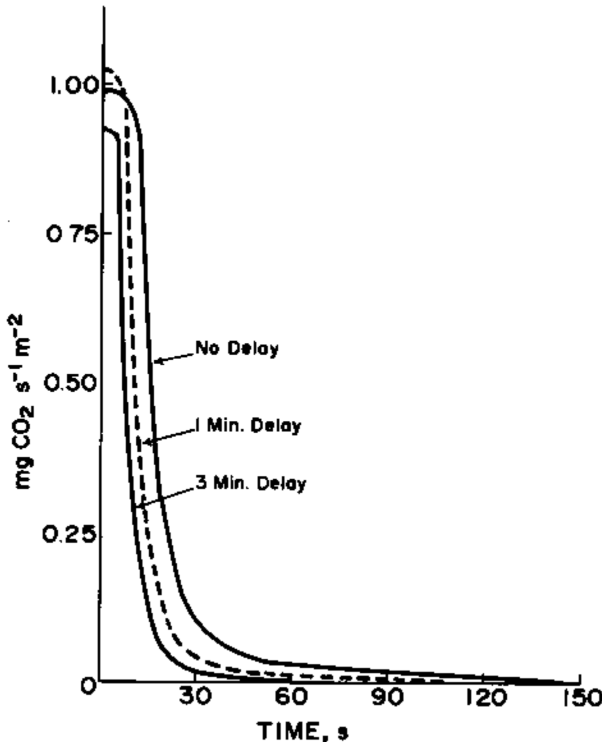

Figure 1. Recorder traces of CO_2 assimilation by a sugar beet leaf following the abrupt onset of darkness. The curve parameters indicate the amount of time the leaf had been in the dark without any external CO_2 supply before the decay curves were measured.

Results and discussion

Figure 1. shows three superimposed recorder traces of CO_2 uptake by a beet leaf as a function of time in the dark. At time zero the light was switched off and the curve labeled 'no delay' was traced. The light was then turned on, the CO_2 uptake allowed to stabilize and then the curve labeled '1 min delay' was traced. This was accomplished by simultaneously turning off the light and blocking the gas flow over the leaf. One minute later the gas flow was resumed at time zero shown in Figure 1 and the dashed curve was traced with the leaf remaining in the dark. The inside curve shows the trace following a three minute dark gas flow delay.

The shape of the three curves shown in Figure 1 may be interpreted by considering how CO_2 is utilized in the chloroplasts. Carbon dioxide and RuP_2 react on specific catalytic sites to form 3-phosphoglycerate. The rate at which CO_2 is fixed depends on the concentrations of CO_2 and RuP_2 as well as the number of activated carboxylation sites. The activation of the

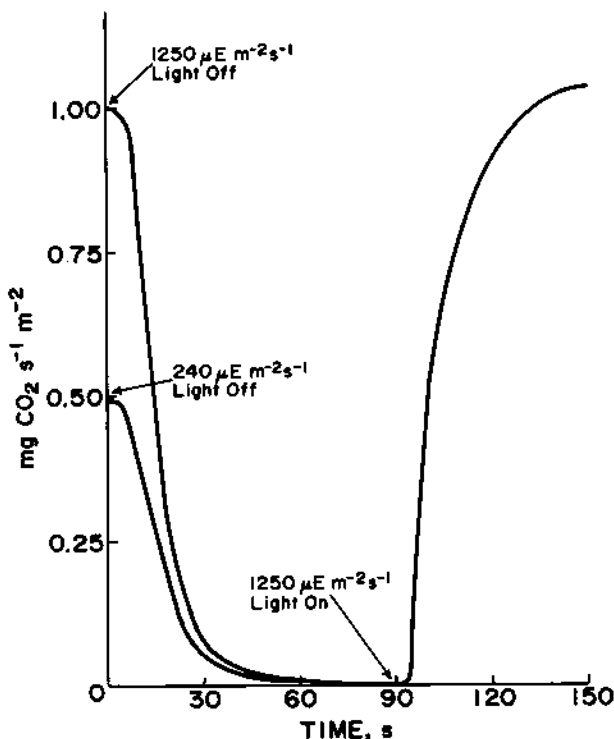

Figure 2. Recorder traces of CO_2 assimilation by a sugarbeet leaf following the abrupt onset of darkness after two steady state light levels. The rise of CO_2 assimilation is also shown following 90 s in the dark.

carboxylation sites and the formation of RuP_2 both require energy orginating from the capture of photons [6]. When the light is turned off the formation of active carboxylation sites stops and their concentration begins to decrease through random disassociation. At the onset of darkness CO_2 fixation continues only until either the pool of RuP_2 or the active carboxylating sites disappear. The reaction of CO_2 and RuP_2 at an active site does not necessarily destroy the site's activity [6].

Consider the consequences of turning of both the light and the gas flow to the leaf and then, after a short delay, resuming gas flow over the leaf in the dark. When the light and gas flow are turned off simulataneously the reaction is soon limited by CO_2 and the rate of use of the residual RuP_2 depends on the amount of CO_2 being formed by respiration. When the gas flow is resumed in the dark the amount of CO_2 subsequently fixed is reduced because the pool of RuP_2 was decreased in the dark by CO_2 from respiration. Note that the areas under the curves shown in Figure 1 (after correcting for instrument lag) represent CO_2 absorbed from the 1% O_2 gas stream in the

dark. Consequently, when there is no delay in the gas flow the amount of CO_2 absorbed is equivalent to the pool size of RuP_2. When there is a delay the pool size of RuP_2 corresponds to the area under the curve plus the amount of CO_2 released by respiration during the time the air stream was stopped.

Could the loss of active carboxylation sites sometimes stop the dark fixation of CO_2 rather than the disappearance of RuP_2? If CO_2 and RuP_2 were both present, and a decrease in active carboxylation sites was forcing CO_2 fixation toward zero in the dark, the initial reaction rate at time zero in Figure 1 would decrease as the dark time delay increased. This would also lead to curves that do not go to zero for at least 3.5 minutes, i.e., because the area under the inside curve in Figure 1 is greater than instrument lag correction, it guarantees that the carboxylation sites remain active at least 3.5 minutes in the dark. The shape of the curves in Figure 1 do then support the proposition that it is the disappearance of RuP_2 rather than the disappearance of carboxylation sites that causes CO_2 assimilation to go to zero following the loss of light. This is in general agreement with other recent results [5, 6].

The coorected area under the dark response curve thus corresponds to the amount of RuP_2 that reacts in the dark. If the light dependent high energy intermediates that form RuP_2 decay within a few seconds following the onset of darkness, the amount of RuP_2 that forms following the onset of dark is small compared to the amount of RuP_2 present initially. We believe this may be a valid assumption, based on the shape of the CO_2 response curves shown in Figure 2. These curves are also similar to those observed by Perchorowicz et al. [5] and Creach and Stewart [3]. The ambient CO_2 level was the same for both curves in Fig. 2 but fixation was reduced by the 'low light'. Low light leads to reduced concentrations of high energy RuP_2 forming intermediates [5]. Thus, if the shape of the dark response CO_2 curves was being significantly affected by the continued formation of RuP_2, the 'high light' curve should not drop off as quickly as the 'low light' curve. However, the 'high light' curve has a steeper slope than the 'low light' curve, indicating the light dependent high energy compounds that drive the formation of RuP_2 must react or decay rapidly. The fast response following the onset of light (Figure 2) supports this interpretation.

Corrected areas are shown in Table 1 for dark time delay measurements of three leaves. The areas decreased to zero on all the leaves after three to five minutes in the dark indicating that the RuP_2 had by that time all reacted with CO_2 produced by dark respiration. The negative area values result from error that may be partly systematic due to the way the instrument lag time correction was made. Leaf dark respiration may cause the CO_2 level in the gas stream to reach the higher steady state level more rapidly than it does over the aluminum foil surface we used for calibration. The random uncertainty in the data we present here is probably near $\pm 20 \mu eq\,m^{-2}$.

Table 1. Effects of withholding CO_2 (0–5 minutes in the dark) on the areas under the subsequent CO_2 assimilation curves and on the time it took these curves to reach a steady state level in the dark.

Leaf fixation rate	Beet 0.97 mg m^{-2} s^{-1}		Beet 1.23 mg m^{-2} s^{-1}		Bean 0.59 mg m^{-2} s^{-1}	
Time delay after light off, min.	decay time s	corrected area μeq. m^{-2}	decay time s	corrected area μeq. m^{-2}	decay time s	corrected area μeq. m^{-2}
0	120	219	76	108	73	245
1	74	144	57	84	49	111
2	60	129	50	30	40	79
3	45	55	37	− 2	36	9
5	36	− 13	31	− 27	26	− 35

The areas in Table 1 may be compared to other measurements of RuP_2 based on the assay of frozen tissue. Perchorowicz et al. [5], Creach and Stewart [3] and Sharkey and Badger [7] reported RuP_2 levels between 150 and 300 mol mg^{-1} chlorophyll (Chl). Assuming 500 mg Chl m^{-2}, the areas in Table 1 of 245 and 108 μeq m^{-2} convert to 490 and 216 nmol mg^{-1} Chl. This suggests the two methods give similar values for RuP_2. Because the RuP_2 pool turnover is so rapid in both the light and dark, it is difficult to obtain representative leaf samples for chemical assay and freeze them before changes occur [7]. There are also possibilities for error in the method we propose here. Already discussed is the likelihood of some RuP_2 formation after the light is turned off, and also the assumption that any change in CO_2 fixation by PEP carboxylase is negligible during the time span of the measurements. We further assume that the dark respiration is constant during the decay periods shown in Figure 1 and 2. If, in fact, it takes 10 or 15 seconds for the dark respiration to rise to a steady state level, the areas we measure would be a little greater than the amount of CO_2 actually used. As previously noted in the methods section the measured rate of CO_2 uptake shown in Figures 1 and 2 is the sum of the net light fixation rate plus the dark respiration rate. Another question concerns the area correction for instrument lag time we used. If the dark evolution of CO_2 does significantly effect the CO_2 time response curve, we may have over-corrected the areas under the curves by as much as 25 μeq m^{-2}. This would be a compensating error compared to the others.

The decay times in Table 1 are measurements of the time required for the curves to fall from their steady state rates of CO_2 uptake in light to their steady state rates in the dark, i.e. to the zero rate shown in Figure 1.

Interpretation of the decay times is not so straight forward as interpreting the areas under the response curves. The decay time depends on characteristics of the gas flow system and the analyzer, as well as the individual concentrations of CO_2, RuP_2, and activated carboxylating sites in the chloroplasts.

When the initial amount of RuP_2 is less, the decay time will be less. On the other hand, a decrease in the number of active carboxylating sites or a decrease in the ambient level of CO_2 in the chloroplasts will increase the decay time. Thus when RuP_2 is less and the decay time does not change, the CO_2 level or the active sites or both must also have decreased. For example, in Table 1 the decay times decreased as the dark delay periods increased. Since the areas, interpreted here as RuP_2 concentrations, also decreased it follows that the activation of RuP_2 carboxylase remained high during the dark periods since the ambient CO_2 was high in the mesophyll tissue. If the decay times had not decreased, or had increased, a logical conclusion would have been that the activity of the carboxylating enzyme was also rapidly decreasing in the dark.

Analysis of CO_2 decay curves has a number of potentially interesting applications. For example, we observed the curves for several leaves that had been fixing CO_2 at steady state levels under saturating and then under limiting light levels. The results are summarized in Table 2. The RuP_2 pool sizes were reduced at low light levels in the case of the two beet leaves. They were not reduced in the bean leaves. The decay times were not really changed by the light, indicating that the activity of RuP_2 carboxylase may also have been reduced by low light intensities, which is in agreement with the results of Perchorowicz et al. [5]. Note the strikingly different relation between changes in RuP_2 concentration and decay times for beets shown in Tables 1 and 2, i.e., the decay times in Table 2 are not proportional to RuP_2 indicating RuP_2 carboxylase was controlling the fixation rate in some cases. The decay times for the last bean leaf in Table 2 was less than those for the first leaf suggesting the activity of the RuP_2 carboxylase was a bit greater in the last leaf.

Table 2. The effect of light levels on the dark decay times and RuP_2 pool sizes associated with various steady state CO_2 assimilation rates, P, and various concentrations of CO_2 in the gas phase of the mesophyll, C_i

Light Leaf	Light $\mu E\ m^{-2}\ s^{-1}$	P $mg\ m^{-2}\ s^{-1}$	C_i $mg\ m^{-3}$	Decay time s	RuP_2 $\mu eq\ m^{-2}$
Beet	1250	1.00	602	77	203
	240	0.49	703	69	97
Beet	1250	1.03	519	76	175
	240	0.53	632	71	111
Bean	1250	0.50	640	96	118
	240	0.32	694	91	105
Bean	1250	0.59	665	73	108
	240	0.32	732	67	114

Another application is shown by the data presented in Table 3 concerning the CO_2 assimilation of a single beet leaf over a 2 day period under a constant light intensity of $1250\,\mu E\ m^{-2}\ s^{-1}$. The first 7 entries refer to measurements

made the first day. They define the CO_2 response curve which is plotted as a dashed line in Figure 3. The pool size of RuP_2 did not appear to be particularly affected by low ambient CO_2 levels; but, do the long decay times indicate that low ambient CO_2 levels reduce the activity of the carboxylating enzymes? The conclusion is not so clear-cut as for the data in Tables 1 and 2 where the concentration of extracellular CO_2 i.e. C_i, remained high. It is possible that, rather than low concentrations of active carboxylating sites, low concentrations of CO_2 in the chloroplasts begin to limit the fixation rate, keeping the decay times large.

On the second day (entries 8 through 14, Table 3) water stress began to develop as shown by the stomatal resistance, r_s. These data also define 'CO_2 response curve' as shown by the arrows in Figure 3. These arrows indicate the progression of measurements during the day. Comparison of the two response curves in Fig. 3 indicate that the decreased CO_2 fixation under water stress was due to factors other than increased stomatal resistance since the use of C_i circumvents dependence on stomatal resistance. C_i was calculated from the approximate relation $P = (C_a - C_i)r_s^{-1}$ where C_a is the ambient concentration of CO_2 in the leaf chamber (Farquhar and Sharkey) [4].

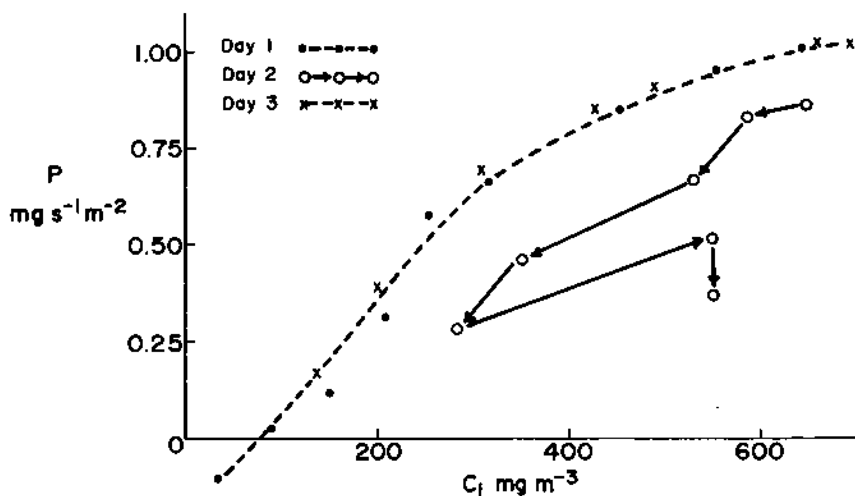

Figure 3. CO_2 assimilation, P, responses of a sugarbeet leaf as affected by CO_2 levels, C_i, in the gas phase of the mesophyll. The solid circles show the response on day one, the arrows and open circles show the response on day 2 as water stress was progressively increasing and the crosses are observations on the 3rd day after watering.

Water stress and the pool size of RuP_2 in the beet leaf did not appear to be singularly related to each other nor did the RuP_2 levels and decay times appear to be much different from the non-stressed measurements on the previous day (Table 3). However, the stomata responded to changes in CO_2

Table 3. Observations of RuP_2 levels and dark decay times for a sugarbeet leaf at various levels of CO_2 assimilations, P, stomatal resistance, r_s, and mesophyll CO_2 concentration, C_i

No.	P $mg\ s^{-1}\ m^{-2}$	r_s $s\ m^{-1}$	C_i $mg\ m^{-3}$	RuP_2 $\mu eq\ m^{-2}$	Decay time s
		not stressed			
1	1.01	120	643	175	73
2	0.95	110	554	147	92
3	0.83	120	449	172	85
4	0.66	110	313	197	102
5	0.57	140	254	156	68
6	0.31	120	209	168	99
7	0.12	110	147	132	102
		Water stress increasing			
8	0.86	270	631	180	89
9	0.83	370	586	191	62
10	0.67	390	525	123	60
11	0.46	290	348	148	66
12	0.28	260	283	134	90
13	0.51	520	557	121	57
14	0.37	740	555	146	72

under stressed conditions (entreis 10–13) but not at these same CO_2 levels under non-stressed conditions (entreis 1–7). Wong et al. [10] have previously cited evidence that stomatal conductance may be coupled with CO_2 assimilation through mechanisms other than stomata gas diffusion resistance. Moreover, interrelations between water stress, abscisic acid, and stomatal response to CO_2 levels have been demonstrated [4, 2, 8] but are not yet completely understood. In any case, the stress effects on stomatal opening and CO_2 assimilation were readily reversible as shown by the CO_2 response points in Figure 3 on the third day when the plant had been watered.

Conclusion

We believe analysis of the dark response curves at 1% O_2 hold considerable potential because it is a nondestructive technique that can provide information on changes in both RuP_2 and RuP_2 carboxylase activity. It is possible that the corrected area under the dark CO_2 response curve over-estimates the initial pool size of RuP_2, but the areas do compare with values of RuP_2 reported in the recent literature. In any case, at low O_2 levels, the area is a measure of the amount of RuP_2 that reacts after the abrupt onset of darkness, and so is at least an indicator of the leaf's inherent ability to produce RuP_2. Refinements in experimental techniques are needed to increase the accuracy of measurements of the corrected areas under light-dark CO_2 response curves. These areas need to be compared to chemical assays of RuP_2 made on adjacent leaves just before the onset of darkness.

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