Plant Physiol. (1989) 89, 1060-1065
0032-0889/89/89/1060/06/$01.00

Received for publication July 12, 1988
and in revised form October 12, 1988

Mild Water Stress Effects on Carbon-Reduction-Cycle Intermediates, Ribulose Bisphosphate Carboxylase Activity, and Spatial Homogeneity of Photosynthesis in Intact Leaves[1]

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ABSTRACT

We have examined the effect of mild water stress on photosynthetic chloroplast reactions of intact *Phaseolus vulgaris* leaves by measuring two parameters of ribulose bisphosphate (RuBP) carboxylase activity and the pool sizes of RuBP, 3-phosphoglycerate (PGA), triose phosphates, hexose monophosphates, and ATP. We also tested for patchy stomatal closure by feeding $^{14}CO_2$. The k_{cat} of RuBP carboxylase (moles CO_2 fixed per mole enzyme per second) which could be measured after incubating the enzyme with CO_2 and Mg^{2+} was unchanged by water stress. The ratio of activity before and after incubation with CO_2 and Mg^{2+} (the carbamylation state) was slightly reduced by severe stress but not by mild stress. Likewise, the concentration of RuBP was slightly reduced by severe stress but not by mild stress. The concentration of PGA was markedly reduced by both mild and severe water stress. The concentration of triose phosphates did not decline as much as PGA. We found that photosynthesis in water stressed leaves occurred in patches. The patchiness of photosynthesis during water stress may lead to an underestimation of the effect of stomatal closure. We conclude that reductions in whole leaf photosynthesis caused by mild water stress are primarily the result of stomatal closure and that there is no indication of damage to chloroplast reactions.

Mild water stress can cause a significant reduction in the rate of photosynthetic CO_2 assimilation in water-stress-sensitive plants such as *Phaseolus vulgaris* (21). While much of the reduction can be attributed to stomatal closure, part of the reduction has been attributed to direct effects of dehydration on the biochemical reactions of photosynthesis (4, 10, 16).

Severe osmotic stress of chloroplasts and cells can inhibit FBPase[2] activity by inhibition of light activation (3) or by lowered pH (2). Other effects of osmotic stress have been reviewed by Kaiser (12), who has persuasively argued that mild water stress does not affect the photosynthetic reactions of the chloroplasts, since photosynthesis of chloroplasts and cells is not inhibited by mild osmotic stresses. For example, photosynthesis of isolated cells was inhibited by 50% by an osmotic stress of −2.0 MPa, but photosynthesis of intact leaves was reduced by 50% when the plant water status fell to −0.8 MPa (24).

Much of the evidence for direct effects of mild water stress on photosynthesis is based on estimates of the partial pressure of CO_2 inside the leaf, which is an attempt to remove the complication of stomatal closure by calculation. In several studies, no effect of mild water stress on CO_2 limited photosynthesis was found (20, 24, 29, 32). However, the rate of photosynthesis at moderately high CO_2 was reduced and was not increased by increasing CO_2 or decreasing O_2. This behavior has been interpreted as an effect on the capacity for starch and sucrose synthesis (22).

Many of the effects of abscisic acid and water stress are similar (5, 14). It has recently been demonstrated that the presumed effects of abscisic acid on the photosynthetic apparatus (15, 19) were caused by closure of stomata in patches of the leaf (7, 28). It is possible that mild water-stress effects on the biochemistry of photosynthesis are an artifact caused by patchy stomatal closure.

The reduction in the rate of photosynthesis induced by water stress must be reflected in a reduction in the rate of the reaction catalyzed by RuBPCase. This can be caused by reduced activity of RuBPCase, or by changes in the availability of either RuBP or CO_2. We have examined the effect of mild water stress on two parameters of RuBPCase activity and the pool sizes of RuBP, PGA, triose phosphates, hexose monophosphates, and ATP. We also tested for patchy stomatal closure to assess the availability of CO_2. A preliminary report of some of these data has appeared (23).

MATERIALS AND METHODS

Plants of *Phaseolus vulgaris* L. (cv Linden) were grown in 4-L pots containing soil in a greenhouse in Reno, Nevada, as described by Seemann and Sharkey (18). Water was withheld until the leaves were mildly stressed (−0.7 MPa), which required an average of 4 d, or severely stressed (−1.1 MPa),

[1] Research supported by U.S. Department of Energy grants DE-FG02-87ER13785 and DE-FG02-87ER60568 to T. D. S. and by U.S. Department of Agriculture Competitive Research Grants Office grant CRCR-1-2470 to J. R. S.

[2] Abbreviations: FBPase, fructose 1,6-bisphosphatase; C_i, partial pressure of CO_2 in the air spaces inside the leaf; FBP, fructose 1,6-bisphosphate; PGA, 3-phosphoglycerate; RuBP, ribulose 1,5-bisphosphate; RuBPCase, RuBP carboxylase/oxygenase (E.C. 4.1.1.39); CABP, 2-carboxyarabinitol 1,5-bisphosphate.

which required 6 d. For ^{14}C labeling the plants were grown in a growth chamber as described by Vassey and Sharkey (31).

Pressure-volume curves were determined by repeated pressure bomb determinations of water potential and leaf weight as leaves dried in air. The water potential of the plants used in each experiment was determined by detaching a small leaf and determining the balancing pressure. All leaves of a given plant were assumed to have the same water potential.

Metabolite pool sizes and RuBPCase k_{cat} and activation state were determined as described by Seemann and Sharkey (18). The RuBPCase activation state is the ratio of activities before and after incubating the leaf extract with CO_2 and Mg^{2+}. (This activity ratio is called carbamylation in Figure 2. N. D. Butz and T. D. Sharkey [unpublished data] have shown that activity ratios are a good measure of carbamylation.) RuBPCase k_{cat} is the catalytic constant of the enzyme in units of mol CO_2 fixed mol^{-1} enzyme s^{-1}, or simply s^{-1}. We report the total activity (after incubating with CO_2 and Mg^{2+}) divided by the enzyme concentration determined by CABP binding.

Patchiness was determined by exposing leaves to $^{14}CO_2$ for 3 min. The specific activity was 3.7 GBq/mol (0.1 Ci/mol) and the partial pressure of CO_2 was 340 μbar. Exposures were carried out in either a small circular gas exchange chamber or in a whole leaf chamber. Leaves were frozen by clamping with the hand-held freeze-clamp or by pressing a large aluminum block, prechilled in liquid N_2, on the leaf. After freezing, the leaves were placed in contact with Kodak SB film and exposures were carried out at −80°C for 5 to 7 d.

RESULTS

Since many of the effects of osmotic stress are related to volume changes, we estimated the volume changes that occurred during water stress of *P. vulgaris* by determining pressure volume curves. A representative pressure volume curve determined with one of the bean plants used in this study is shown in Figure 1. We assumed that the points lying on a straight line indicated the change in osmotic pressure as water was lost (30). A linear regression of these points is shown as a dotted line in Figure 1. From this regression we estimated the osmotic pressure at each water potential. From the changes in osmotic pressure we calculated that the mild water stress treatment caused a 4% reduction in cell volume. More severe stress (−1.1 MPa) caused a 20% reduction in volume. Direct measurements of relative water content were not made during this study, but are reported for this variety of bean in Vassey and Sharkey (31).

We equilibrated leaves of control, mildly stressed and severely stressed plants in air in our gas exchange chamber/freeze clamp. The mildly stressed leaves had photosynthetic rates which were approximately 50% of control rates (Fig. 2). The severely stressed leaves had negligible rates of photosynthesis in air. The availability of CO_2 was reduced in the mildly stressed leaves but the estimated intercellular CO_2 partial pressure was very high in the severely stressed leaves because of the very low rates of photosynthesis. The k_{cat} of RuBPCase which could be measured after incubating the enzyme with CO_2 and Mg^{2+} was unchanged by water stress (Fig. 2) indicating that production of carboxyarabinitol 1-phosphate did not occur during water stress (17). The ratio of activity before and after incubation with CO_2 and Mg^{2+}, reported as carbamylation in Figure 2, was somewhat reduced by severe stress but not by mild stress. Likewise, the RuBP pool size was somewhat reduced by severe stress but not by mild stress.

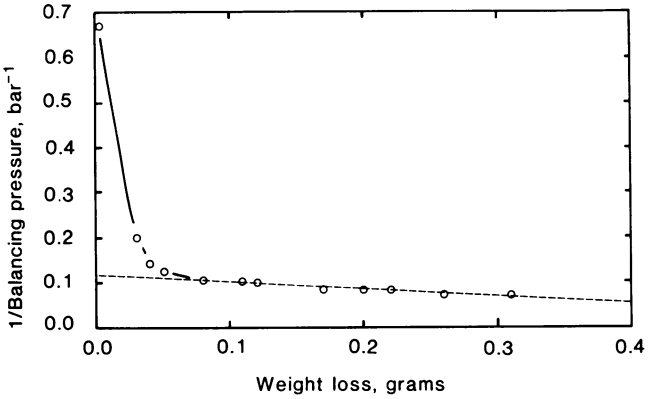

Figure 1. Pressure-volume curve for a leaf of *P. vulgaris*. After allowing the cut end of the petiole to stand in water overnight, the balancing pressure was determined in a pressure bomb and the leaf was weighed. After drying for a short period in air the leaf was weighed and the balancing pressure was determined again. The dashed line is a linear regression through eight data points.

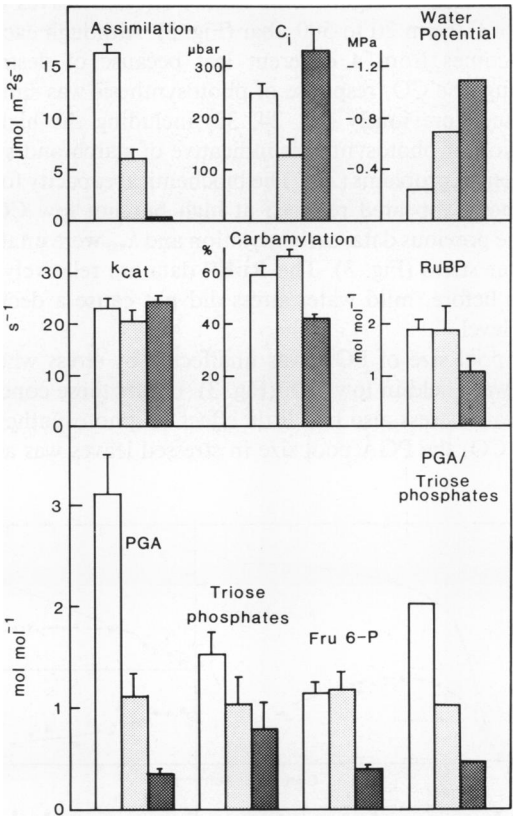

Figure 2. Assimilation rate, water potential and biochemical measurements of leaves of *P. vulgaris*. The open bars are control data, the light stipled bars are data from mildly stressed plants (−0.7 MPa) and the dark stipled bars are data from severely stressed leaves (−1.1 MPa). Concentrations are reported as mol mol^{-1} RuBPCase binding sites as determined by CABP binding.

In contrast, the pool size of PGA was markedly reduced by both mild and severe water stress (Fig. 2). The pool size of triose phosphates did not decline as much as PGA in response to water stress. The level of FBP was just at our limits of detection (0.2 mol mol^{-1}) but no tendency toward large FBP pools was detected (data not shown). If triose phosphates and FBP were anywhere near aldolase equilibrium, then the reduced pool size of triose phosphate in the water stressed plant indicates that pool size of unbound FBP was also reduced. The level of Fru 6-P was unchanged by mild water stress but reduced by severe stress (Fig. 2). These measurements of metabolite pool sizes all represent whole leaf levels of these compounds. This presents no problem in interpreting the RuBP data and little problem in interpreting the PGA data. However, changes in hexose monophosphates in the stroma can be masked by opposite changes in the cytosol (TD Sharkey, P Vanderveer, unpublished data).

We next asked whether the decline in C_i could account for the decline in photosynthesis in response to mild stress. To answer this question, we examined many of the same parameters reported in Figure 2 in mildly stressed leaves over a range of C_i from 20 to 500 μbar (Fig. 3). Although each data point comes from a different leaf because of destructive sampling, the CO_2 response of photosynthesis was similar to those seen previously (22, 24, 32) including the high CO_2 inhibition of photosynthesis indicative of starch and sucrose metabolism problems (22). The biochemical capacity for photosynthesis appeared reduced at high but not low CO_2. As with the previous data, carbamylation and k_{cat} were unaffected by water stress (Fig. 3). The RuBP data are relatively noisy but, as before, mild water stress did not cause a decline in RuBP level.

The pool size of PGA was unaffected by stress when the leaves were held in low CO_2 (Fig. 3). Under these conditions mild water stress also had little effect on photosynthesis. At higher CO_2 the PGA pool size in stressed leaves was as little as 20% of the controls. The level of ATP declined as CO_2 was increased but there was no apparent effect of mild water stress on ATP level. Although the PGA pool in the stressed leaves was as low as 20% of the control level, the triose phosphate pool sizes were unaffected by water stress. Fru 6-P was slightly higher in controls than stressed leaves but the ratio of Glc 6-P/Fru 6-P was higher in stressed leaves.

Overall, it appeared that the reduced rate of photosynthesis caused by water stress could be accounted for by reduced CO_2 availability, though our estimates of intercellular CO_2 indicated that the CO_2 was present. We therefore tested whether our estimates of intercellular CO_2 could be in error during water stress because of patchy stomatal closure, as has been found in abscisic acid-treated leaves. When unstressed leaves were exposed to $^{14}CO_2$, the uptake of label was uniform over the leaf. However, water stressed leaves took up label in patches (Fig. 4). These patches were usually limited by veins, indicating that these veins prevent gas diffusion from one aerole to the next (21). The patchiness was specifically associated with acute water stress and was not visible in control leaves or one day after rewatering the plants. The lack of label in the veins indicate that very little movement of photosynthate occurred during the three min labeling period.

DISCUSSION

The mild water stress imposed in these studies inhibited photosynthesis while causing only a small reduction in cell volume. The reason for the very small reduction in cell volume in response to the mild stress is that as turgor is lost, relatively small changes in volume cause large changes in water potential. The severely stressed leaves were just past the point of wilting. At this point the water potential is equal to the osmotic potential of the cell sap. When cells or chloroplasts are rapidly osmotically stressed, photosynthesis is close to maximum at −1.1 MPa but when plants dry out to −1.1 MPa over six days, photosynthesis is all but absent. It is

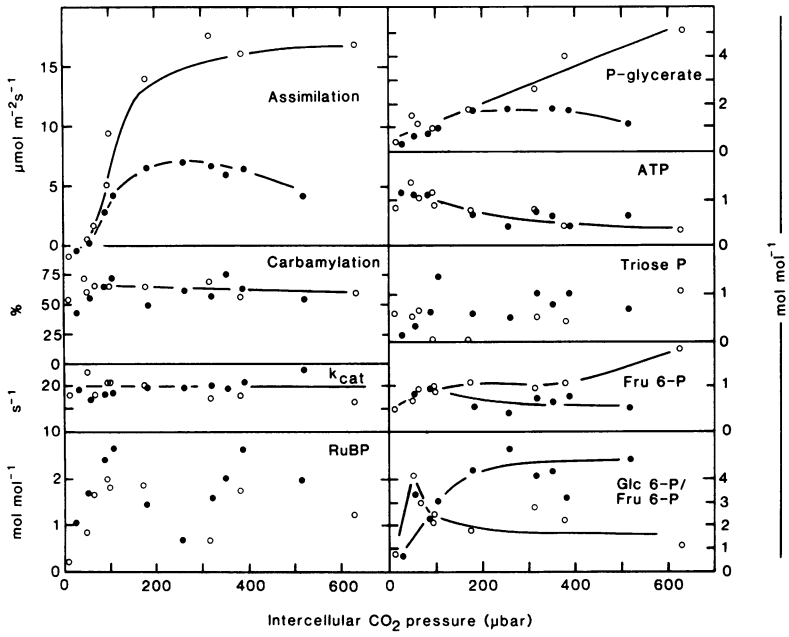

Figure 3. Assimilation rate and biochemical measurements of leaves of *P. vulgaris*. The open symbols are control values and the filled symbols are data points for plants stressed to −0.7 MPa. Concentrations are reported as mol mol^{-1} RuBPCase binding sites as determined by CABP binding.

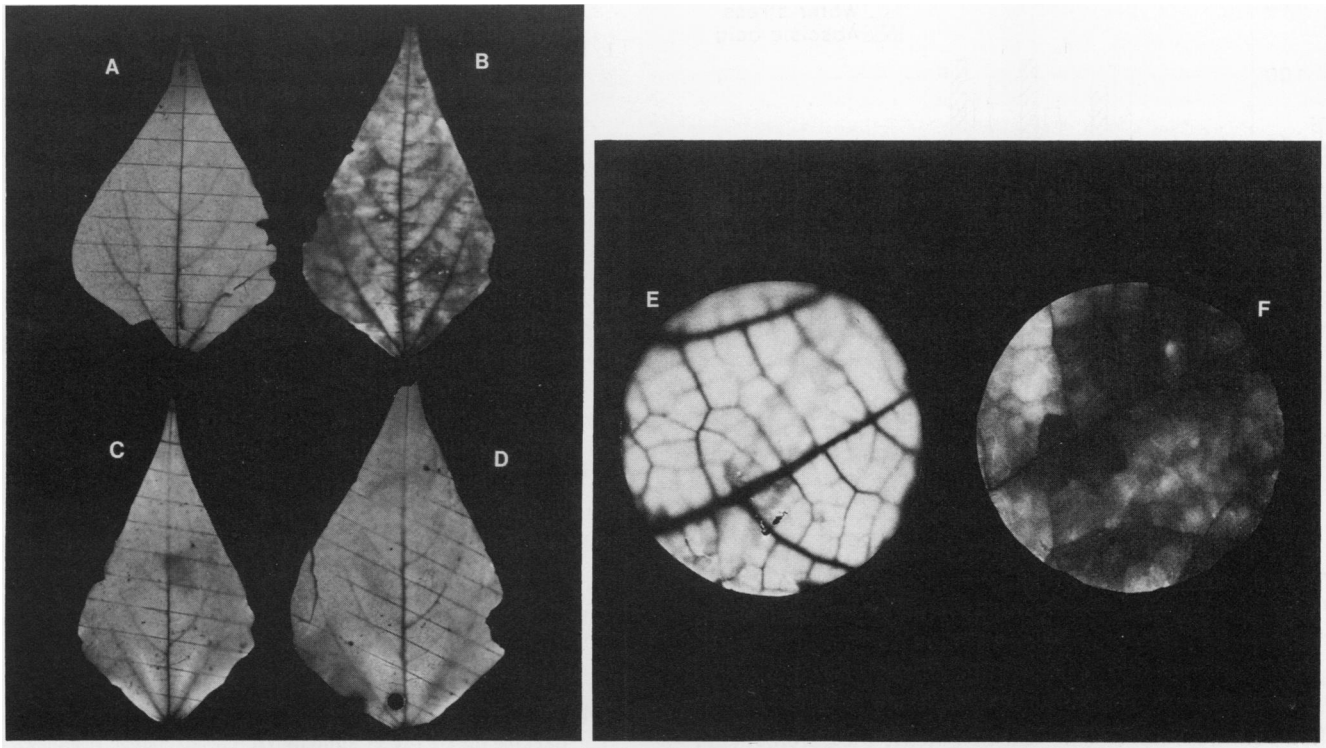

Figure 4. Autoradiograms of leaves allowed to photosynthesize in the presence of $^{14}CO_2$ for 3 min and then frozen. A, C, and E are control leaves, B is a mildly water-stressed leaf, panel D is leaf 1 d after rewatering and F is an enlarged view of a water-stressed leaf piece showing that photosynthesis varied from one aerole to the next. Autoradiograms were used as negatives so that light areas indicate CO_2 uptake. The exposures were varied to give approximately equal overall densities between treatments to emphasize the distribution of photosynthesis across the leaf. The parallel lines on the whole leaf autoradiograms were caused by shadows of fishing line used to secure the leaf in the exposure chamber.

unlikely that volume changes, which were small, could account for the almost complete disappearance of photosynthesis in intact leaves.

Mild water stress had no effect on the carboxylation capacity of RuBPCase. Both carbamylation ratio and k_{cat} of the enzyme were unchanged by mild water stress (Figs. 2 and 3), indicating that the activity of RuBPCase was not affected by regulatory mechanisms known to affect this enzyme under other circumstances (17). If enzymes of the photosynthetic carbon reduction cycle other than RuBPCase, specifically FBPase, were the primary site of water stress effects, then the RuBP level should fall in response to stress. The concentration of RuBP did not decline (Figs. 2 and 3), nor was there an indication that the pool of FBP was building up. We conclude that the effects of water stress on FBPase occur at more severe stress than imposed in this study.

In mild stress, the rate of RuBP consumption (measured as whole leaf CO_2 assimilation) was reduced by 50%, but the pool of RuBP did not increase (Figs. 2 and 3). This result indicates that there may be feedback control on the level of RuBP, preventing the buildup of excessive amounts of RuBP. The constancy of metabolite concentrations despite large changes in photosynthetic rate indicates that the regulatory mechanisms of photosynthetic carbon metabolism were not damaged by water stress. Since we measured whole leaf levels of metabolites, compensating changes in levels of metabolites found in both the chloroplast and cytosol (triose phosphates, hexose phosphates and ATP) could obscure some effects. However, most of our arguments are based on RuBP concentrations and RuBP is present only in the chloroplast.

We saw no decline in the ATP level of mildly stressed plants. The ratio of PGA to triose phosphate declined with water stress (Fig. 2), also indicating that energy input into the photosynthetic carbon reduction cycle was not reduced by water stress, as has been reported by Dietz and Heber (6). Other investigations have demonstrated little or no photoinhibition during mild water stress (1, 13, 26).

In summary, we found no evidence for a lesion in the chloroplast biochemistry necessary for photosynthesis induced by mild water stress. Nevertheless, photosynthesis was reduced from control levels at equal estimated intercellular CO_2 partial pressure.

Photosynthesis in water-stressed bean leaves occurs in patches (Fig. 4), as has been described for abscisic acid fed leaves (7, 28). The changes in enzyme activity and metabolite levels induced by water stress are nearly identical to the changes caused by abscisic acid (Fig. 5) (5, 8, 14, 19). The indication that abscisic acid affects the biochemistry of photosynthesis is now believed to be an artifact resulting from the patchy stomatal closure induced by abscisic acid (7, 28). Since patchiness also occurs in water stressed plants (Fig. 4) (11, 27) we conclude that the major effect of mild water stress

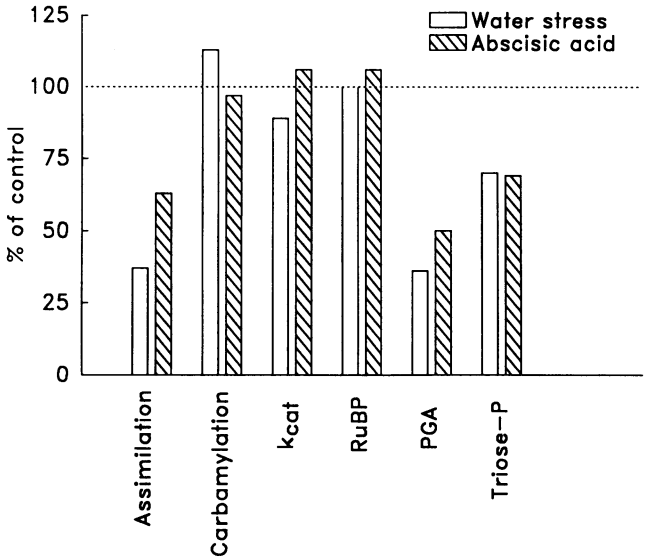

Figure 5. Comparison of mild water stress effects (data from this study) with abscisic acid effects (data from Ref. 19).

on photosynthetic CO_2 assimilation is stomatal closure, not a reduction in the capacity of the chloroplasts for photosynthesis. Because the closure can be patchy, measurements of gas exchange may be unreliable during water stress. The reports of transpiration effects on photosynthesis depended upon accurate estimations of intercellular CO_2. These reports (9, 20) may be in error in light of the results presented here.

The lack of O_2 inhibition of photosynthesis during mild water stress (22, 31) and lack of CO_2 stimulation (22, 31, 32) is not explained by patchy stomatal closure or abscisic acid effects. Photosynthesis in leaves fed abscisic acid almost always will increase if the CO_2 partial pressure is increased (15, 21). The loss in sensitivity induced by water stress can be caused by a loss in enzyme capacity for sucrose synthesis as demonstrated for the FBPase lacking mutant of *Flaveria linearis* (25). Vassey and Sharkey (31) have found that mild water stress reduces starch synthesis and the extractable activity of sucrose phosphate synthase, which would cause a loss O_2 inhibition of photosynthesis.

In summary, we believe that mild water stress reduces photosynthesis in intact leaves primarily by causing stomatal closure, in agreement with early work by Troughton and Slayter (29). We agree with Kaiser (12) that the chloroplast photosynthetic reactions are not affected by mild water stress. The effects of mild water stress on the relationship between photosynthesis and intercellular CO_2 partial pressure, which have been interpreted to indicate effects of mild water stress on chloroplast metabolism, can be explained by the effects of mild water stress on starch and sucrose metabolism (31) and by the patchy closure of stomates.

ACKNOWLEDGMENTS

Bob Bowden built the chamber used to expose whole leaves to $^{14}CO_2$. We thank Judy Miles for technical assistance with the metabolite analyses in Reno and Peter Vanderveer for technical assistance with the autoradiography in Madison.

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