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# Guard Cell Starch Biosynthesis Regulated by Effectors of ADP-Glucose Pyrophosphorylase[1]

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## ABSTRACT

**ADP-glucose pyrophosphorylase catalyzes the regulated step of starch bioynthesis in mesophyll chloroplasts. This enzyme is activated by a high ratio of the concentrations of 3-P-glycerate to inorganic phosphate (Pi) in light. In contrast, starch in guard cell chloroplasts is degraded when stomata open, which usually occurs in light. We have investigated the biochemical causes for this contrasting phenomenon.**

***Vicia faba* L. leaflets were sampled in darkness and after various periods of illumination. The samples were quick-frozen and freeze-dried. Guard cells and other cells were dissected out, weighed, and assayed for ADP-glucose pyrophosphorylase activity, 3-P-glycerate, and Pi. In the pyrophosphorolytic direction, ADP-glucose pyrophosphorylase specific activity in guard cells was 2.7 moles per kilogram protein per hour, which was comparable to the values obtained for palisade and spongy cells. The specific activity in epidermal cells was 4-fold lower. Under our assay conditions, the guard cell enzyme activity was 5-fold higher in the presence of 3-P-glycerate and 5-fold lower with Pi (*i.e.* similar to the results obtained with extracts of fresh leaflet). During three minutes of illumination, 3-P-glycerate concentration in palisade cells increased 2.5-fold to 10 millimoles per kilogram dry mass. The concentration of 3-P-glycerate in guard cells was 20-fold lower and unaffected by illumination. The concentration of Pi was approximately 17 millimoles per kilogram dry mass in palisade cells, but was 10-fold higher in guard cells. These overall cellular Pi concentrations were unaffected by illumination. We conclude that starch biosynthesis in guard cells is not activated by light because of the low and constant 3-P-glycerate concentration there. We interpret this last to be a consequence of the absence of the photosynthetic carbon reduction pathway in chloroplasts of these cells.**

Typically, stomata open when a leaf is illuminated. During stomatal opening, guard cell starch concentration declines (14), and organic anions accumulate (11, 13). Although the entire pathway has not been documented, it is probable that starch is metabolized to hexose-bisP, which is oxidized to PEP[2] through glycolysis. P-enolpyruvate is carboxylated (11, 12, 26) to form oxalacetate, which is converted to more stable anions. In normal mesophyll cells, illumination also has numerous effects; one is the activation of ADP-glucose pyrophosphorylase (23), the regulated step in starch biosynthesis. This activation is brought about by high [PGA] to [Pi] ratio in light (23). In brief summary, starch synthesis is favored in mesophyll cells in light (when these cells are active in carbon reduction), in marked contrast to starch degradation in guard cells in light.

We speculated (15) that the difference in starch metabolism between guard cells and mesophyll cells may be due to the absence of ribulose-bisP carboxylase in guard cells (3, 9, 11, 15, 19, 26). The activity of this enzyme is thought to be the dominant source of elevated PGA concentration in light. Although this hypothesis was reasonable, support for it was equivocal: massive carbon flux through glycolysis that occurs in guard cells during stomatal opening might intrinsically elevate intermediates of this pathway, including PGA. Therefore, we have determined the effect of light on PGA and Pi concentrations in guard cells and palisade cells. We also have measured the activity of ADP-glucose pyrophosphorylase in guard cells and determined its sensitivity to PGA and Pi. In other current work (25), the kinetic properties of this guard cell enzyme are reported in more detail.

## MATERIALS

Fully expanded bifolate leaves of *Vicia faba* L. were used in all experiments (see Ref. 19 for culture conditions). Analytical enzymes were from Boehringer; most other biochemicals were from Sigma.

## GENERAL PROTOCOL

For metabolite estimation, plants were placed in darkness for 30 min and then illuminated (~400 $\mu E \cdot m^{-2} \cdot s^{-1}$). At indicated times, a cork borer was used to take samples from individual leaflets. The isolated samples (which had remained under illumination during harvesting) were quenched rapidly in $N_2$ (~−215°C; cooling $N_2$ to its melting point by evacuation increases the tissue cooling rate by reducing the Leidenfrost phenomenon). For ADP-glucose pyrophosphorylase activity measurements, preilluminated, whole leaves were quenched in $N_2$. Samples were dried ≥48 h at 10 $\mu$m Hg and −35°C. (The usual additional precaution of slowly warming the samples to room temperature while under vacuum was also taken.) For storage, the samples were maintained under vacuum at −20°C. For analysis, individual cell samples were dissected out, weighed on a quartz fiber balance (5–15 ng), and then extracted in sub-$\mu$l droplets under oil. All assays were coupled to the oxidation/reduction of pyridine nucleotides, which were subsequently amplified enzymically. The final reaction indicator was measured fluorometrically. For details of the methodology, see Lowry and Passonneau (8) and Outlaw (10).

Prior to microanalysis, the assay procedures were validated and optimized with whole leaf extracts (*cf* Ref. 12). We emphasize that the reference basis for all experiments was mass determination of individual cell samples. For the sake of comparison, protein bases also have been calculated (16); a considerable error in this conversion is possible with epidermal cell samples. In addition, Chl bases were calculated from Chl:protein ratios found earlier with isolated palisade cells (18) and guard cell protoplasts (17). As Pi is distributed throughout the cell (1), conversion of metabolite data to cell volume bases (21) was made. Most PGA

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[2] Abbreviations: PEP, P-enolpyruvate; PGA, 3-P-glycerate.

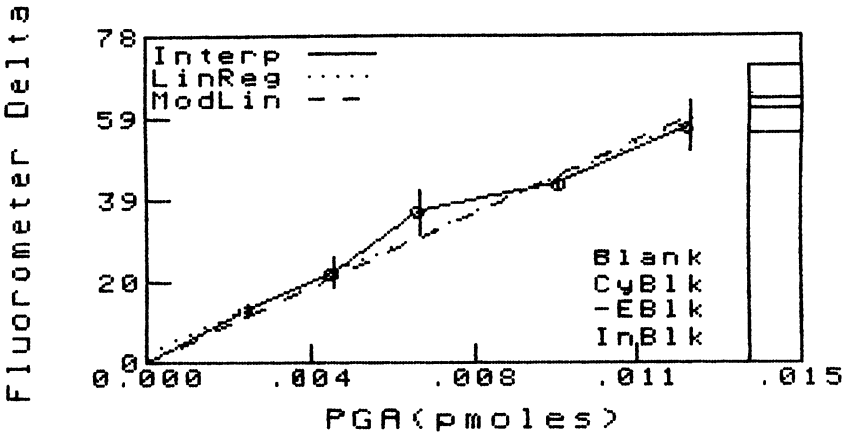


FIG. 1. Typical standard curve for PGA (2–12 fmol). Different lines are for linear regression, interpolation, and modified linear regression (*i.e.* artificially 'driven' through zero). Bars are SE ($n$ = 3 for each datum). Various blank values (see Ref. 8) are shown in the histogram inserted in the right side. (Blank, complete blank; CyBlk, enzyme cycling blank; −EBlk, as blank except specific step analytical enzyme was deleted; InBlk, indicator reagent blank).

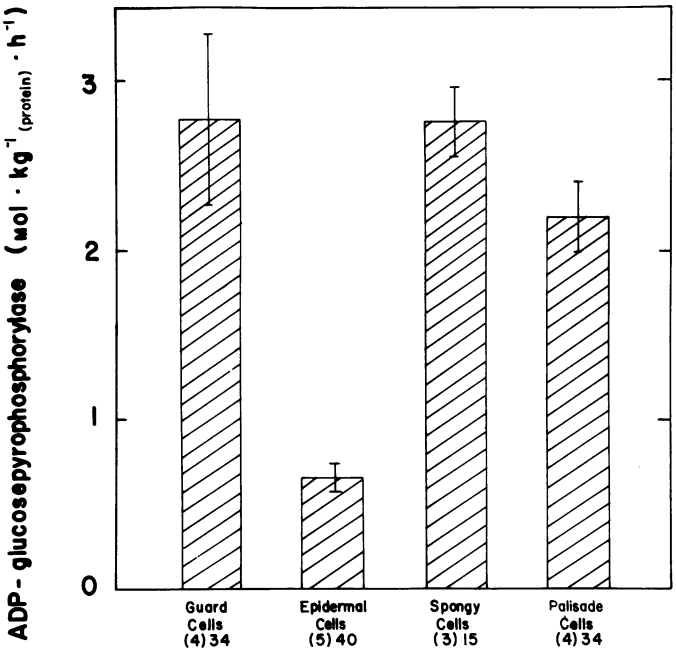


FIG. 2. Histological distribution of ADP-glucose pyrophosphorylase in *Vicia faba* leaflet. Enzyme activity was assayed in the pyrophosphorolytic direction using quantitative histochemical methodology. Bars are SE. The number of experiments (in parentheses) and total number of tissue samples are shown below the cell identification.

Table I. *Effect of PGA and Pi on ADP-Glucose Pyrophosphorylase Activity in Individual Guard Cell Pairs*

SE/mean ≈ 0.15 for each activity.

| | Enzyme Activity | | |
|---|---|---|---|
| | Control[a] | − 3-P-glycerate | + 2 mM Pi |
| | *mmol/kg dry mass·h* | | |
| Exp. 1 ($n$ = 23) | 35.7 | 7.7(22)[b] | 5.9(17) |
| Exp. 2 ($n$ = 25) | 35.0 | 5.9(17) | 10.9(31) |

[a] Contained 1 mM PGA.
[b] Numbers in parentheses, % of control.

in light is chloroplastic (27), so metabolite data for both palisade and guard cells were converted also to a chloroplast volume basis using the Chl:volume ratios of mesophyll chloroplasts measured by Heldt and Sauer (5) and the Chl content of guard cells (17) or palisade cells (calculated earlier). (Unfortunately, variability in reports of guard cell chloroplast volumes, dimensions, and numbers precluded their use (*e.g.* Ref. 20 *versus* 21). We recognize considerable errors may accumulate in these derivative calculations, so interpretation of the derived values must be made with caution.

### ADP-GLUCOSE PYROPHOSPHORYLASE

**Procedure for Activity Measurements.** *Step 1.* The reaction was initiated by addition of sample to 0.5 μl (guard cells and epidermal cell pieces) or 2 μl (palisade and spongy cells) of 50 mM Hepes (pH 7.4) containing 0.5 mM DTT, 1 mM PGA, 0.2 mM EDTA, 0.02% w/v BSA, 10 mM KF, 10 mM $MgCl_2$, 0.3 mM PPi, 0.1 mM $NADP^+$, 4 μg/ml glucose-6-P dehydrogenase (from yeast, EC 1.1.1.49), 6 μg/ml P-glucomutase (from rabbit muscle, EC 2.7.5.1), 0.5 μM glucose-1,6-$P_2$, 0.8 mM ADP-glucose (deleted for tissue blanks), and, for standardization in 'blank' wells, various concentrations of NADPH or glucose-1-P, as appropriate.

*Step 2.* After 60 min at ~22°C, the reaction was terminated by addition of 0.5 μl (or 2 μl) of 0.2 N NaOH.

*Step 3.* The oil well rack was heated to 80°C for 20 min (to destroy $NADP^+$), and then NADPH was amplified as usual (8).

**Procedure for Sensitivity to PGA and Pi.** Step 1 of the above procedure was altered in the following ways: (a) 100 mM Tris-HCl (pH 8.5) was substituted for 50 mM Hepes (pH 7.4); (b) PGA was omitted, as indicated; (c) 2 mM Pi was added, as indicated; (d) ADP-glucose concentration was 0.1 mM.

**Comments.** The assay principle is the conversion of ADP-glucose to glucose-1-P (2). Prior to histochemical experiments, the assay was validated and the cocktail was optimized using extracts of fresh leaflet. Additional control experiments verified that leaf preparation for histochemical analysis was without effect. The altered cocktail used to determine effects of PGA and Pi was based on kinetic studies of fresh leaf extract; this altered cocktail resulted in lower velocity, but increased sensitivity.

### PHOSPHATE

The procedure of Lowry and Passonneau (8) was followed. This assay is based on the Pi requirement in the enzymic conversion of glycogen to hexose-P.

### PGA

**Procedure.** *Step 1.* The sample was added to 7 nl (for half-palisade cells, ≈6 ng) (or 2 nl [for guard cell pairs, ≈6 ng]) of 0.02 N NaOH.

*Step 2.* The extracts were heated to 60°C for 20 min.

*Step 3.* Seven nl (or 2 nl) of reagent was added to give an assay cocktail containing 50 mM K-phosphate (pH 7.0), 10 mM DTT, 1 mM $MgCl_2$, 20 mM NaCl, 0.3 mM ATP, 0.02% w/v BSA, 50 mM ascorbate, 1 mM EDTA, 0.1% w/v PVP, 30 μM NADH, ~10

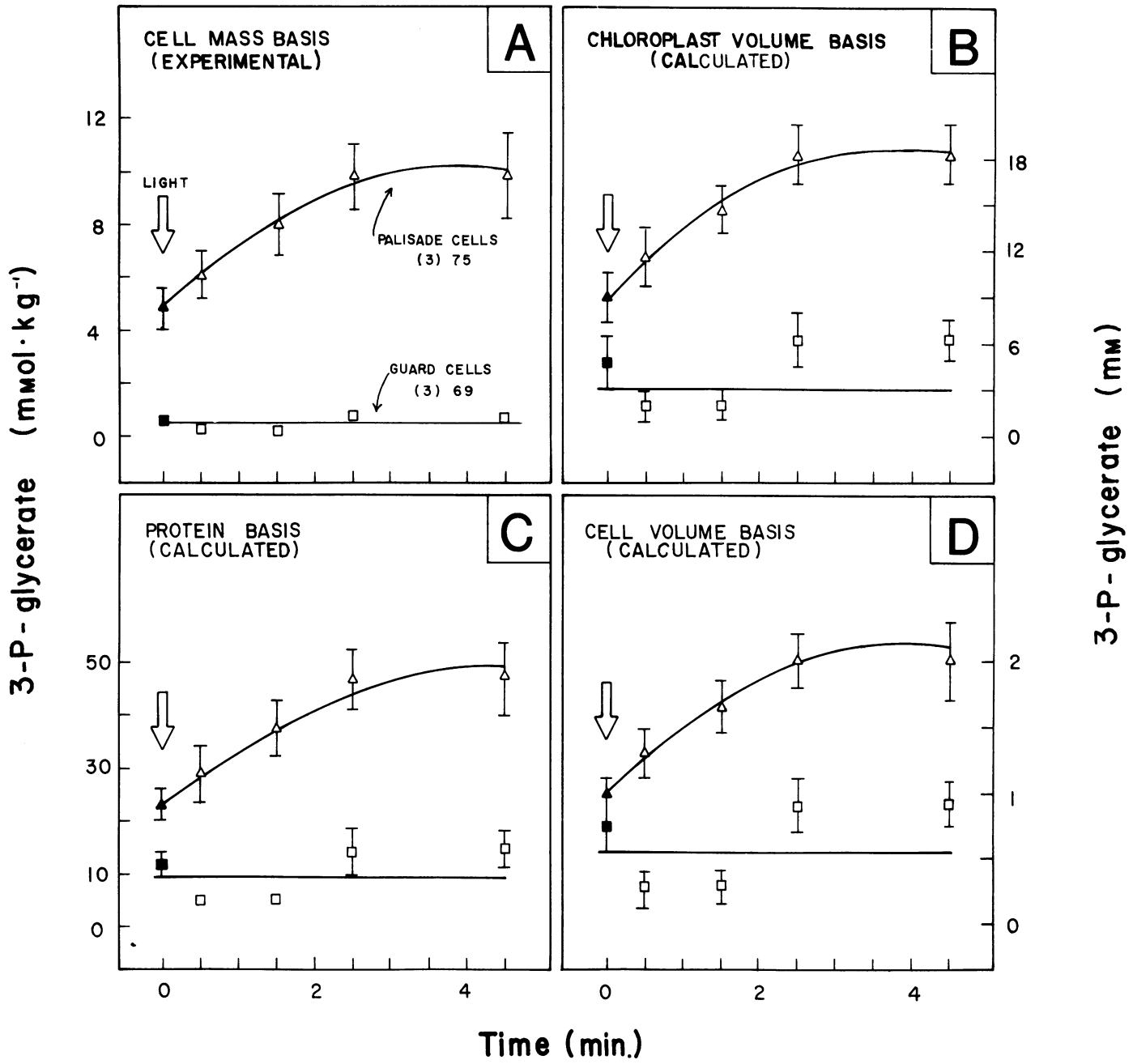


FIG. 3. PGA concentration in palisade cells (△, ▲, three experiments, $n = 75$) and guard cells (□, ■, three experiments, $n = 69$) of *Vicia faba* leaflet determined at various times following the onset of illumination. (Samples for the darkened symbols were taken immediately before illumination.) For each experiment, samples were taken from an individual leaflet. The samples were rapidly quenched and dried. Individual guard cell pairs and palisade cells were dissected, weighed, and assayed for PGA. (Pi also was assayed in cells of some of these same samples [Fig. 4]). Panel A shows experimental values; other panels show derived values; see "Materials and Methods." Bars are SE.

μg/ml dialyzed glyceraldehyde-P dehydrogenase (from yeast, EC 1.2.1.12—deleted for tissue blanks), ~5 μg/ml dialyzed P-glycerate kinase (from yeast, EC 2.7.2.3), and, for standardization in 'blank' wells, various concentrations of $NAD^+$ and PGA.

*Step 4.* After 30 min at 22°C, 7 nl (or 2 nl) of 0.3 N HCl was added (to destroy NADH).

*Step 5.* After the oil well rack was heated to 60°C for 20 min, $NAD^+$ was enzymically amplified as usual (7).

**Comments.** The principle is the reduction of PGA to glyceraldehyde-P (8). The specificity of NADH oxidation for PGA was verified by comparison of the reaction kinetics for authentic PGA with those of $HClO_4$ extracts of fresh leaflet. Special precautions were required to estimate such small quantities of PGA accurately. Thus, PVP, DTT, and ascorbate were found empirically to lower blank values (presumably by preventing nonspecific oxidation of NADH, a problem encountered in small volumes). Heat destruction was at 60°C (instead of the usual 80°C) to lower evaporative loss through the oil. A standard curve for PGA, which shows the precision of the assay and various blank values, is presented in Figure 1.

## RESULTS

ADP-glucose pyrophosphorylase activity was 2 to 3 mol/kg protein · h in photosynthetic parenchyma cells and guard cells. By contrast, this activity was 4-fold lower in samples of epidermal

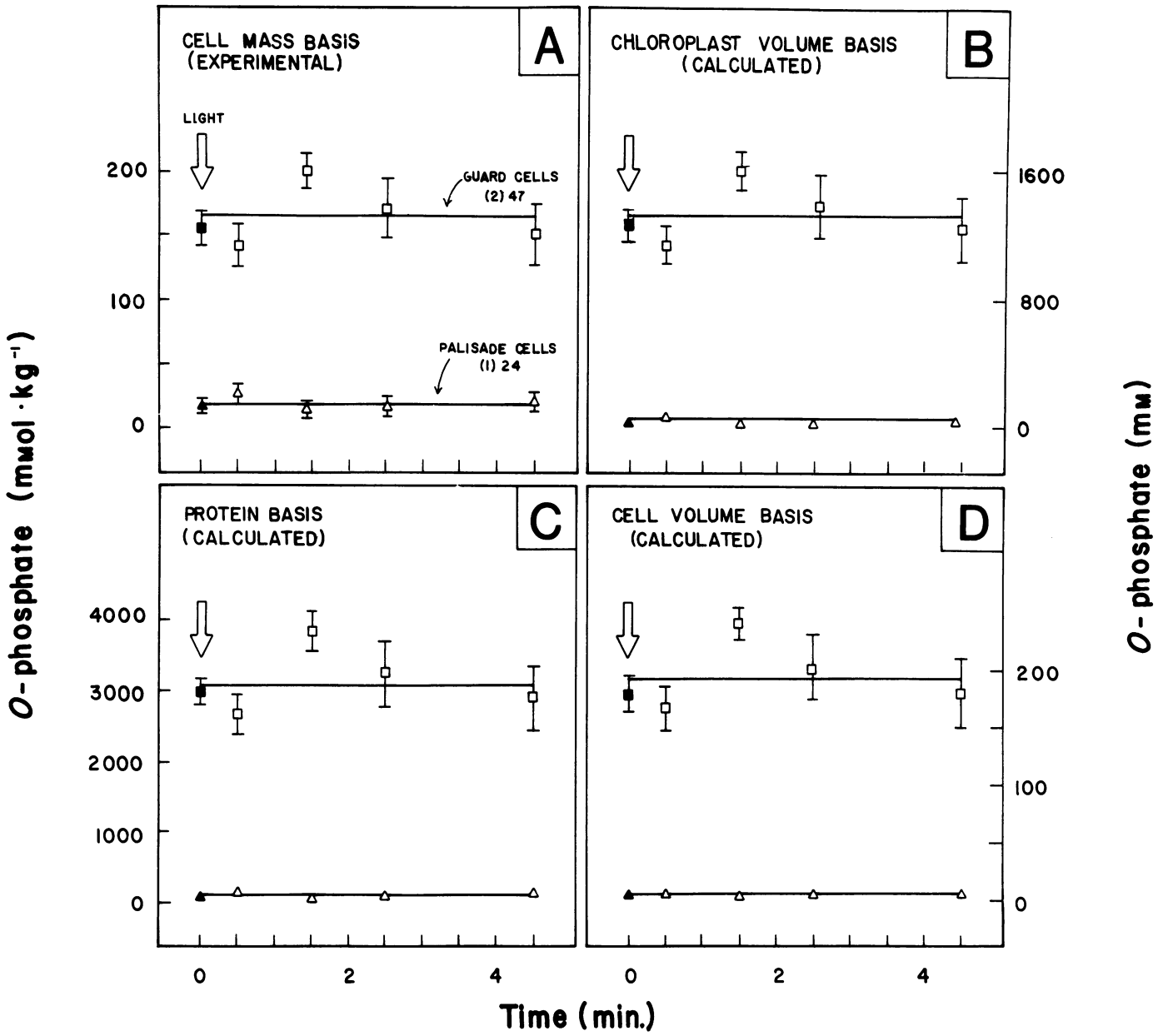


FIG. 4. Pi concentration in palisade cells ($n$ = 24) and guard cells ($n$ = 47). Other details are as in Figure 3.

cells (Fig. 2).

*Vicia* leaflet ADP-glucose pyrophosphorylase had a higher velocity in the presence of PGA and a lower velocity in the presence of Pi (data not shown). This regulation was more pronounced at higher pH (= 8.5) and low ADP-glucose concentration (= 0.1 mM), but the velocity was diminished about 4-fold. Similarly, under these conditions, inclusion of 1 mM PGA in the assay cocktail elevated guard cell ADP-glucose pyrophosphorylase activity by 5-fold (Table I). Inclusion of 2 mM Pi in the assay cocktail containing PGA decreased the activity by approximately 5-fold (Table I).

Guard cell PGA concentration (≈0.5 mmol/kg dry mass) (Fig. 3A) was considerably lower than that in palisade cells (≈4–10 mmol/kg dry mass) (Fig. 3A). The concentration of PGA in guard cells was unaffected by illumination (Fig. 3A), even after 30 min (data not shown). However, palisade cell PGA concentration increased 2.5-fold within 3 min after illumination. These general results (relatively different concentrations, different effects of light) were unchanged if the data were converted to other bases (Fig. 3, B, C, and D).

On mass basis, Pi concentration in guard cells was 175 mmol/kg dry mass (Fig. 4A). In marked contrast, Pi concentration in palisade cells was less than 20 mmol/kg dry mass (Fig. 4A). At the precision of these measurements, Pi concentration was unaffected by illumination in both these cell types (Fig. 4A). Regardless of the basis upon which the data are expressed, Pi concentration was higher in guard cells than in palisade cells (Fig. 4, B, C, D).

## DISCUSSION

Upon illumination, starch accumulates in whole leaf; the concentration of starch in guard cells declines during this time (see Introduction). In order to understand why these differences occur, we initially have studied starch biosynthesis in guard cells and palisade cells. Several possibilities, which might cause these differences, are discussed below.

First, there is the unlikely possibility that the starch biosynthetic pathway in guard cells does not include the step catalyzed by ADP-glucose pyrophosphorylase. However, we report (Fig. 2) ADP-glucose pyrophosphorylase activity in guard cells of *Vicia* to be 2.7 mol/kg protein · h (measured pyrophosphorolytically), and Robinson *et al.* (25) report the activity in *Commelina* guard cells to be 1.8 mol/kg protein · h (measured by ADP-glucose synthesis). The difference in starch content between guard cells of open and closed stomata was 1.3 mol/kg protein (anhydroglucosyl units, Ref. 14). (Unfortunately, quantitative kinetic studies have not been published.) Thus, although our data do not rule out a role for phosphorylase there is no reason to expect an anomaly: ADP-glucose pyrophosphorylase seems adequate to replenish guard cell starch reserves during normal stomatal movements. Our first conclusion is that starch biosynthesis in guard cells and other leaf cells involves the same pathway (*cf.* 24).

Second, there is the possibility that guard cell ADP-glucose pyrophosphorylase is less sensitive or insensitive to the effectors that regulate this activity in other leaf cells. (ADP-glucose pyrophosphorylases from diverse biological sources may be classified by their specificity/selectivity toward metabolites for activation [see Ref. 22]). For leaf ADP-glucose pyrophosphorylase, the primary activator is 3-P-glycerate, while the inhibitor is Pi (see Introduction). Our results (Table I) show these metabolites are effective in regulating guard cell ADP-glucose pyrophosphorylase, in a fashion similar to the regulation of the enzymes in extracts of fresh *Vicia* leaflet. Likewise, Robinson *et al.* (25) report the *Commelina* guard cell enzyme also is regulated by these effectors. As expected, these metabolites had a greater effect in their assay system (toward ADP-glucose synthesis) than in ours (*cf.* Ref. 22). Our second conclusion is that guard cell and mesophyll cell ADP-glucose pyrophosphorylases have the same regulatory properties.

Third, it is possible that the concentrations of ADP-glucose pyrophosphorylase effectors differ in different cell types and/or are affected differently by illumination. Following illumination of *Vicia* leaflets, we found the total PGA pool to increase to 195 nmol/mg Chl (data not shown). This value is approximately twice the value of spinach chloroplastic PGA (6). On mass basis, PGA concentration in palisade cells was 3-fold higher than in whole leaflet (Fig. 3), a result that is consistent with the finding that most PGA is restricted to chloroplasts (27). However, our total cellular PGA concentration, when simply calculated to chloroplast volume basis, was 4.5-fold higher than the values measured by Kaiser and Bassham (6) in spinach chloroplasts. Despite the overestimation inherent in our conversions, it appears that a quantitative difference exists between our data and those of Kaiser and Bassham (6). This difference may result from physiological state (isolated organelle *versus* intact tissue), species differences, or other causes. More importantly, our results on palisade cells dissected from whole leaves are in agreement with the general finding that the PGA concentration increases following illumination of photosynthetic systems. In sharp contrast to the situation with palisade cells, illumination had no effect on cellular PGA concentration in guard cells (Fig. 3), even after 30 min (when starch to malate conversion is occurring [12]). These results show directly (a) the relationship of the photosynthetic carbon reduction pathway to the metabolite pool size changes, and (b) that PGA does not increase in concentration in guard cells during the metabolism of starch to malate. An additional finding (Fig. 3) is that PGA concentration was considerably lower in guard cells than in photosynthetic palisade cells, regardless of the basis upon which the data are expressed. Thus, our third and most important conclusion is that starch biosynthesis in *Vicia* guard cells is not activated when these cells are illuminated because of low and constant PGA concentration therein, *i.e.* the dissimilarity of PGA concentration and pool size kinetics between guard cells and palisade cells may be sufficient to explain how these cells regulate starch biosynthesis differently.

We also measured Pi concentration in guard cells and palisade cells. We did not detect an effect of illumination in either cell type, perhaps because most Pi is extra-chloroplastic. Thus, from our data on whole cells (Fig. 4), we can neither make inferences concerning chloroplastic Pi nor make interpretations beyond those previously reviewed (4). However, we note the overall concentration of this inhibitor to be much higher in guard cells than in palisade cells. To our knowledge, such striking histological compartmentation of Pi has not been reported previously. Possibly, this information will be important to other investigations of plant metabolism. For this reason, we have extended our studies to other leaf cell types and used different techniques, which rely on different assay principles. These additional measurements will be reported elsewhere (W. Outlaw, M. Tarczynski, W. Miller, unpublished data).

In summary, guard cell starch biosynthesis is not activated by light, because illumination does not result in elevated PGA concentrations in these cells, which would increase the PGA to Pi ratio and activate ADP-glucose pyrophosphorylase. From one perspective, this answer is a negative one. It will be important to determine when starch is synthesized and why. A complete answer requires that starch degradation also be studied.

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