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Enzymic and Substrate Basis for the Anaplerotic Step in Guard Cells[1]

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ABSTRACT

From the maximum rate of malate accumulation in *Vicia faba* L. guard cells during stomatal opening the maximum rate of organic anion synthesis is calculated to be 200 millimoles per kilogram dry weight per hour. A minimum estimate for the phosphoenolpyruvate (PEP) carboxylase-catalyzed reaction in guard cells is 650 millimoles per kilogram dry weight per hour which is significantly higher than in any other leaf tissue. The apparent K_{mPEP} of the guard cell enzyme is 60 μM at pH 8.7, but is probably higher at lower pH. The concentration of PEP in guard cells was 270 μM (=2.2 × 10^{-15} moles/guard cell pair) during anion synthesis. These results support the possibility that the carboxylation of PEP is the anaplerotic step in guard cells.

Gas exchange between leaves and the atmosphere occurs through stomata on leaf surfaces. Stomata open and close in response to environmental stimuli. Aperture increases when the pair of guard cells surrounding the stoma becomes turgid. Turgidity is achieved mostly by influx of K^+ ions (2–4) which are exchanged for protons. Internal charge and pH are stabilized, in part, by the generation of protons during the accumulation of Krebs cycle anions (1, 11). Net anion synthesis requires an anaplerotic step. Using sensitive quantitative histochemical techniques applied to single pairs of guard cells dissected from *Vicia faba* leaflet, we have investigated the enzyme and substrate basis for this step.

MATERIALS AND METHODS

Details for plant culture and sample preparation (7), malate measurement (11), and general histochemical techniques and fluorometry (10) have been published. Sample purity has been documented previously (7, 11).

The enzymes, supplied by Boehringer as a $(NH_4)_2SO_4$ suspension, were freed of most SO_4^{2-} by centrifuging, discarding the supernatant, and dissolving the pellet in 25 mM Tris-HCl (pH 8.1) containing 0.02% (w/v) BSA. Other biochemicals were from Sigma. Stock solutions of PEP,[2] NADH, and aspartate were enzymically standardized in a spectrophotometer (10).

TISSUE PROTOCOL FOR MALATE ACCUMULATION AND PEP CONCENTRATION

Young fully expanded leaflets of *V. faba* L. were excised while the petioles were submerged under water. The petioles were maintained under water for the course of the experiment. Stomata were caused to close by placing the leaflets in darkness for 1.5 hr. At the beginning of the experiment, the leaflets were placed in light and reduced CO_2 tension (11). At various times during stomatal opening, leaflets were quick frozen and freeze-dried. Guard cell pairs were dissected out, weighed, and assayed for malate. PEP was assayed in guard cells in which malate was accumulating (30 min after stomatal opening was induced; see Fig. 4).

PHOSPHOENOLPYRUVATE ASSAY

The specificity of the following coupled enzymic steps were used to assay PEP:

$$\text{ADP} + \text{PEP} \xrightarrow[\text{pyruvate kinase}]{Mg^{2+},\ K^+} \text{pyruvate} + \text{ATP} \quad (1)$$

$$\text{NADH} + \text{pyruvate} \xrightarrow[\text{lactate dehydrogenase}]{} \text{NAD}^+ + \text{lactate} \quad (2)$$

Whole Leaflet Analysis. Three-hundred mg of leaflet was plunged into liquid N_2 slurry and then powdered with a mortar and pestle at −80 C. The powder was transferred to a centrifuge tube at −10 C and 2 ml of 3 M $HClO_4$ (also at −10 C) was added. After constant stirring for 15 min, the tube was warmed to 2 C and centrifuged at 5,000*g* for 10 min. To 1 ml of the supernatant (about 2.6 N acid) was added 1.5 ml of previously titrated 2 M $KHCO_3$ (concentration adjusted to give a final pH of 6.8). $KClO_4$ was removed by centrifugation at 5,000*g* for 10 min. Fifty mg of activated charcoal was mixed into the extract and then removed by centrifugation. Extracts were used immediately or stored at −80 C. Extracts with added authentic PEP showed no loss during this procedure nor did extracts tan on warming to room temperature. (One M $HClO_4$ was found to be insufficient to precipitate all *Vicia* protein as judged by darkening of extracts. This could be prevented with 1 M DTT.)

One hundred fifty μl of the extract was added to 1 ml of reagent containing 50 mM sodium phosphate buffer (pH 7.0), 50 mM KCl, 10 mM DTT, 1 mM $MgCl_2$, 100 μM ADP, 1.5 μM NADH, 0.02% (w/v) BSA. After an initial determination of fluorescence was made, 10 μg of lactate dehydrogenase (from rabbit muscle, EC 1.1.1.27) was added. After the decrease in NADH fluorescence due to pyruvate and other possible lactate dehydrogenase reacting substances was determined, 1 μg of pyruvate kinase (from rabbit muscle, EC 2.7.1.40) was added. After 10 min, the decrease in fluorescence due to the PEP was measured. Quenching of NADH fluorescence by plant extract was assessed by comparison of internal standards of PEP to the standard curve for PEP (0.1–1.0 × 10^{-9} mol).

Microanalysis. To assay PEP in single frozen-dried guard cell pairs, the following principles were used: (*a*) Tissue enzymes and NAD^+ were destroyed by heating in alkali. (*b*) Endogenous

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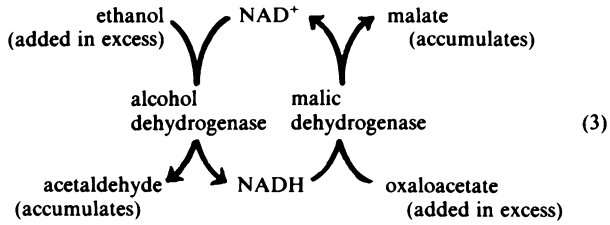
[2] Abbreviation: PEP: phosphoenolpyruvate.

pyruvate was trapped by hydrazine (steps 1–3). (*c*) After the specific step (step 4), excess NADH was destroyed by acid leaving NAD^+ equal to the amount of PEP reacted (step 5). (*d*) NAD^+ was enzymically amplified according to the cycling method of Kato *et al.* (8):



NAD^+ was alternately reduced and oxidized, resulting in the accumulation of thousand-fold amounts of acetaldehyde and malate (steps 6 and 7). (*e*) The accumulated malate was oxidized with additional NAD^+ (step 8):

$$NAD^+ + \text{malate} \xrightarrow[\text{malic dehydrogenase}]{} \text{oxaloacetate} + NADH \quad (3)$$

$$\text{Oxaloacetate} + \text{glutamate} \xrightarrow[\text{glutamic-oxaloacetic transaminase}]{} \text{aspartate} + \alpha\text{-ketoglutarate} \quad (4)$$

Reaction 4 was used to "pull" reaction 3. (*f*) Excess NAD^+ was destroyed without the production of fluorescent product(s) by heating in weak alkali (step 9). (*g*) NADH was oxidized (by H_2O_2) and converted to a highly fluorescent product by heating in strong alkali (step 10). Imidazole rendered this fluorescent product light-stable (9).

Step 1. One and five-tenths nl of 0.04 N NaOH containing 10 mM hydrazine (solution pH = 12.0) was delivered into oil wells with a constriction pipette ("oil well technique," ref. 10). Standards of PEP in NaOH and hydrazine were carried through all of the steps of the assay.

Step 2. Guard cell pairs were pushed onto the alkali droplets.

Step 3. The oil well rack was heated to 60 C for 20 min.

Step 4. After the rack cooled to room temperature, 1.5 nl of specific step reagent was added to the oil well droplet. The reagent was 100 mM sodium phosphate (pH 6.3), 2 mM $MgCl_2$, 100 mM KCl, 10 μM NADH, 20 mM DTT, 200 μM ADP, 0.04% (w/v) BSA, 15 μg/ml lactate dehydrogenase, and 2 μg/ml pyruvate kinase. Incubation was for exactly 30 min at 23 C.

Step 5. Fifty nl of 0.1 N HCl was added to each reaction droplet.

Step 6. After 10 min, 1 μl of cycling reagent (with 3 mM oxaloacetate) was added to each well. Incubation was for 20 hr at 3 C. Enzyme composition was 300 μg/ml alcohol dehydrogenase and 30 μg/ml malate dehydrogenase.

Step 7. Cycling was stopped by adding 0.2 μl of 0.3 N HCl and heating to 80 C for 20 min.

Step 8. One μl of the reaction solution was transferred from the oil well rack to a fluorometer tube containing 40 μl of the malate dehydrogenase-glutamate oxaloacetate transaminase reagent of Kato *et al.* (8). NAD^+ concentration of this reagent was reduced to 100 μM. Incubation was for 30 min at 23 C.

Step 9. Forty μl of 0.3 M sodium phosphate buffer (0.15 M Na_2HPO_4, 0.15 M Na_3PO_4) was added to each tube. Incubation time was 20 min at 60 C.

Step 10. One ml of alkali reagent (6 N NaOH, 10 mM imidazole, 10 mM H_2O_2) was added to each tube. Incubation was for 15 min at 60 C.

Step 11. After the tubes were cooled to room temperature, the fluorescence was determined.

Comments on the Assay. Reaction kinetic data for authentic PEP and leaf extracts were similar, showing that enzymic NADH oxidation in the specific step was a true measure of PEP.

The ability to measure such miniscule amounts of PEP depends on reducing blank values to acceptable limits while maintaining reproducibility. It was possible to reduce the specific step reagent blank to the equivalent of 0.7 μM PEP (=2.1 × 10^{-15} mol in 3 nl [Fig. 1]) by taking the following precautions: (*a*) stock NADH solutions were made in carbonate buffer (pH 10.6) and heated immediately prior to use to destroy NAD^+ present. (*b*) Nonspecific oxidation of NADH was reduced by DTT. Generally, nonspecific oxidation of NADH was only encountered when small volumes were used. For example, oxidation was not measurable after an hr at 23 C in 1 ml of phosphate buffer, but in 10 nl 24% was oxidized. DTT was more effective than ascorbic acid or 2-mercaptoethanol in preventing oxidation; cysteine was ineffective. Volume-dependent rate of oxidation was not affected by choice of buffer (imidazole, phosphate, and a series of Good buffers). However, imidazole in the presence of DTT caused oxidation of NADH even in large volumes. (*c*) Monosodium ADP was made in 2 equivalents of NaOH solution (pH 11.5), heated to 95 C for 10 min, and neutralized with HCl. This treatment destroyed possible contaminating NAD^+ present in ADP. (*d*) Lactate dehydrogenase and pyruvate kinase were dialyzed overnight against 25 mM Tris-HCl (pH 8.1). The blank from the cycling step was 0.4 nM (=0.5 × 10^{-15} mol) and the blank from the malate oxidation step was 0.4 × 10^{-15} mol. The total blank was 3.3 × 10^{-15} mol. The standard error of the difference for the fluorescence values for the blank and the values for standard amounts of PEP at the level present in guard cells was 3 fluorometer divisions. The absolute difference was 10-fold greater.

Destruction of tissue NAD^+ in the 0.04 N NaOH and 10 mM hydrazine (step 1) was complete after 20 min at 60 C. To provide a margin for error, it would generally be preferable to heat to 80 C, however, heating the 1.5 nl (=0.0015 μl) droplets at 80 C resulted in evaporative loss of most samples.

A test with 100 μM pyruvate showed that the reaction with hydrazine was complete; reversal of the reaction was not detected after 30 min. Lactate dehydrogenase in the specific step was in large excess so that the pyruvate formed (equation 1) would be reduced before reacting with hydrazine (10).

PHOSPHOENOLPYRUVATE CARBOXYLASE ASSAY

Whole Leaflet Analysis. Phosphoenolpyruvate carboxylase (orthophosphate: oxaloacetate carboxylase [phosphorylating] E.C. 4.1.1.31) was assayed by determining the PEP-dependent formation of oxaloacetate in the presence of tissue extract, Mg^{2+}, and HCO_3^-.

Extracts were prepared by grinding 0.3 g of leaflet in 5 ml of 50 mM Tris-HCl (pH 8.1), 1 mM DTT, and 2% (w/v) insoluble PVP at 4 C. The homogenates were treated with 20 mg/ml of activated charcoal. Cell debris, PVP, and charcoal were removed by centrifugation at 5,000*g* for 10 min. Extracts were used within 2 hr of preparation.

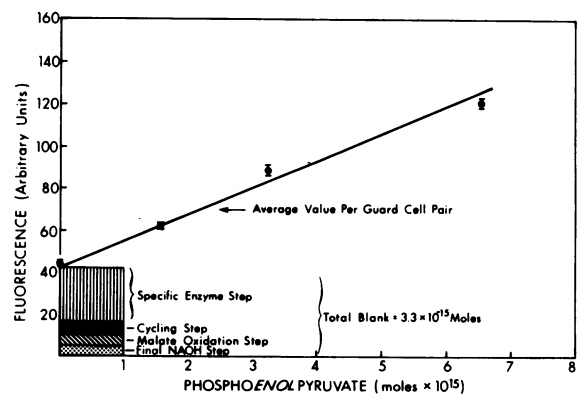


FIG. 1. Standard curve for PEP used for guard cell analysis. Error bars are ± 1 SE for four replicates at each level.

One to 10 μl of extract was used to initiate the reaction in 1 ml of reagent consisting of 50 mM Tris-HCl (pH 8.7 unless otherwise indicated), 1 mM PEP (except as noted), 1 mM $MgCl_2$, 10 μM NADH, 0.5 mM $NaHCO_3$ and 10 μg/ml malate dehydrogenase (from pig heart, EC 1.1.1.37). PEP-dependent oxidation of NADH was measured fluorometrically. Assay reagent pH given is the calculated value based on a pK of 8.1 and was 0.2 units higher than indicated by electrode. Enzyme activity was saturated with respect to HCO_3^- concentration at all pH values.

Microanalysis. Single guard cell pairs and other small freeze-dried tissue samples were also assayed for PEP carboxylase activity by determining the PEP-dependent formation of oxaloacetate. Because of possible problems of instability of oxaloacetate, it was trapped by transaminating to aspartate in the reaction mixture. After the incubation was terminated by heat, the oxaloacetate was released by reverse transamination and assayed utilizing the same general procedures used to assay PEP but on a much larger scale.

Step 1. Tissue samples were pushed onto 1-μl droplets of reagent in oil wells. The reagent was 50 mM Tris-HCl (pH 8.7), 1 mM $MgCl_2$, 0.5 mM $NaHCO_3$, 100 μM glutamate, 0.02% (w/v) BSA, and 20 μg/ml of glutamate-oxaloacetate transaminase (from pig heart, EC 2.6.1.1) and typically 1 mM PEP. Tissue blanks contained no PEP.

Step 2. Typical incubation time was 1 hr at 25 C.

Step 3. The reaction was terminated by heating to 80 C for 20 min.

Step 4. One μl of aspartic reagent (100 mM imidazole [pH 6.2], 15 μM NADH, 50 μM α-ketoglutarate, 2.5 μg/ml of malate dehydrogenase, and 10 μg/ml of glutamate-oxaloacetate transaminase) was added to the oil well droplet.

Step 5. After 15 min at 23 C, 1 μl of 0.3 N HCl was added to the droplet.

Step 6. After 5 min, a 2-μl aliquot was transferred from the oil well rack to 50 μl of enzyme cycling reagent (1,000 cycles, ref. 8) in a fluorometer tube. The tubes were incubated for 1 hr at 25 C.

Step 7. Cycling was stopped by heating to 95 C for 3 min.

Step 8. One ml of indicator reagent (8) was added to each tube.

Step 9. After 30 min incubation at 23 C, the fluorescence was measured.

Comments on the Assay. The microassay was technically more difficult than the macro version. Consequently, the assay was optimized by evaluating various parameters with whole leaf extract. This logic assumes that the guard cell enzyme is identical to whole leaf enzyme; this is not necessarily true as several kinetically distinct PEP carboxylases are known in plants (14, 15). Therefore, the reported activities should be considered minimum values. The reported activities are not the maximum attainable because of the limitation of the indirect methods used which required a long incubation period. For example, initial rates with whole leaf homogenate were 35% greater at 40 C than at 25 C, but enzyme instability was evident during 1 hr of incubation above 35 C. In addition, the pH response of the whole leaf enzyme suggests that the maximum velocity in guard cells would be even higher if assayed at lower pH.

This is the first report of enzyme quantification in single plant cells, therefore data are presented which show linearity of product formation with time and tissue concentration (Figs. 2 and 3). To evaluate these parameters, several assays were conducted with the incubation time varied (Fig. 2) or with one or two guard cell pairs per reagent droplet (Fig. 3).

RESULTS

The maximum rate of malate accumulation in guard cells (110 mmol/kg dry wt·hr) occurred at about 30 min after the leaflets were placed in light and reduced CO_2 (Fig. 4). Little malate appeared to be synthesized during the initial phase of stomatal opening.

The affinity of whole leaf PEP carboxylase for PEP increased with pH from 7.2 to 8.7, but the V_{max} was greatest at pH 8.2 (Table I). The rate with low PEP levels (proportional to V_{max}/K_m) increased 75-fold from pH 7.2 to pH 8.7 (Table I).

A histogram of PEP carboxylase activity in *Vicia* leaflet is

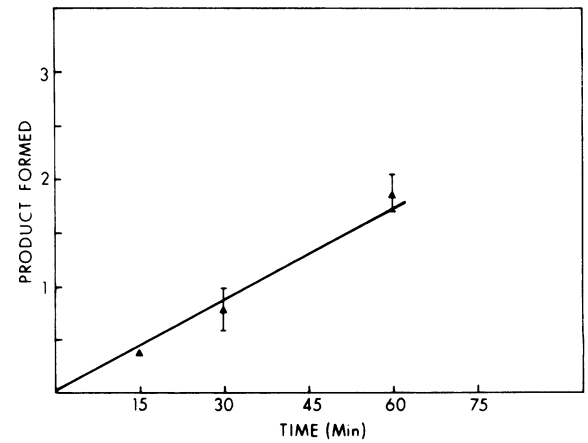


FIG. 2. Product formed by PEP carboxylase in guard cells during various reaction times. Error bars are ± 1 SE for about 10 samples at each point.

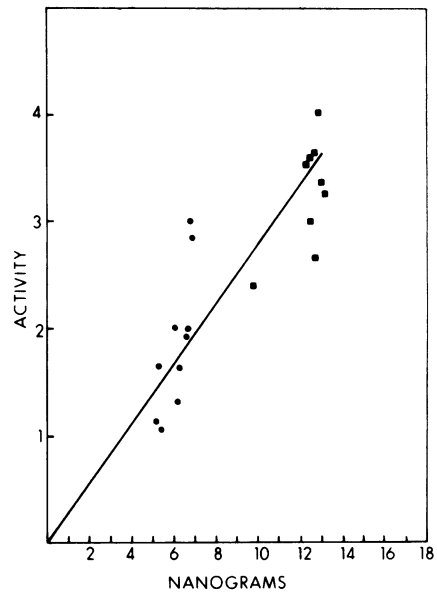


FIG. 3. PEP carboxylase activity in one guard cell pair (●) or two guard cell pairs (■).

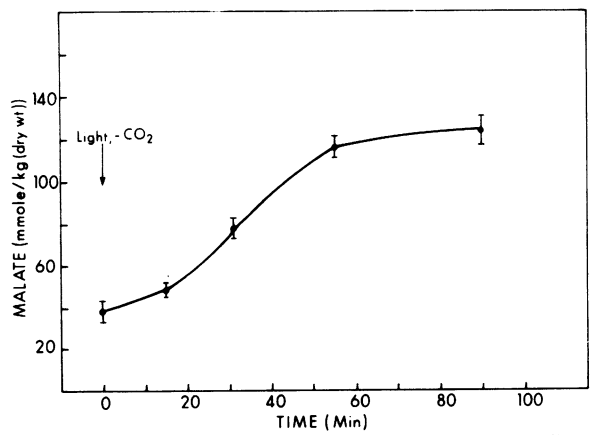


FIG. 4. Time course for malate accumulation in guard cells. Closed stomata were induced to open by light and reduced CO_2. Error bars are for about seven samples at each point.

shown in Figure 5. Activity (25 C, pH 8.7) in guard cells was about 650 mmol/kg dry wt·hr and was 2- to 3-fold higher in guard cells than in other leaf tissue analyzed. In a replicate experiment with a different leaflet, results similar to those shown were obtained, except activity in spongy cells was almost as high as in guard cells. In four experiments with different leaflets, the activity in guard cells was always at least twice the activity in palisade cells.

TABLE I. Effect of pH on V_{max} and apparent $Km_{(PEP)}$ of PEP carboxylase in leaf extract.

pH	V_{max} (relative)	Apparent $Km_{(PEP)}$ (mM)
7.2	68%	4.5
7.7	83%	0.4
8.2	100%	0.2
8.7	64%	0.06

Mg^{2+} was constant at 1 mM.

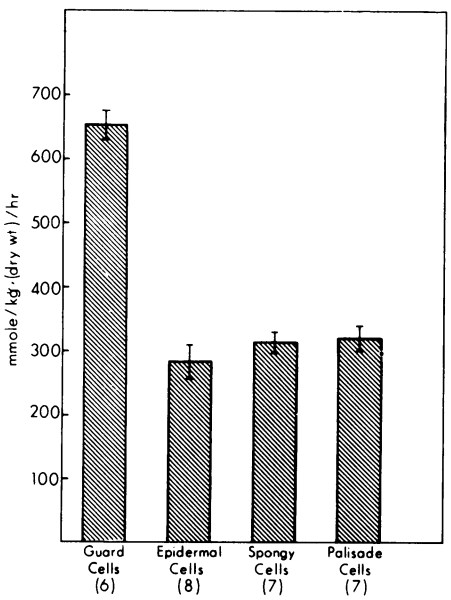


FIG. 5. Histogram of PEP carboxylase activity in *Vicia* leaflet. Bars are ± 1 SE for the number of samples shown in parentheses below the histogram.

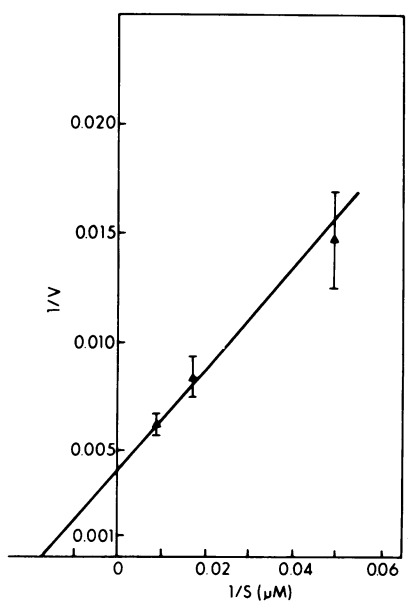


FIG. 6. Double reciprocal plot of guard cell PEP carboxylase activity at pH 8.7 and PEP concentration. This gives a K_m of 60 μM. Error bars indicate ± 1 SE for about 10 samples each.

TABLE II. PEP concentration in guard cells.

$mmol/kg_{(dry\ wt)}$ ± SE	moles/guard cell pair	estimated internal concentration[1]
0.31 ± 0.06 (n = 9)	2.21×10^{-15}	0.27 mmolar

[1] The concentration was calculated using a guard cell volume estimated to be 8 pl. This volume is substantially less than expected from guard cell dry mass (∿ 6 ng) probably owing to the thick walls of guard cells.

K_{m}PEP of guard cell PEP carboxylase at pH 8.7 was 60 μM (Fig. 6) which did not differ from that of whole leaf (Table I).

PEP concentration in whole leaflet was 0.49 mmol/kg dry wt. This value is comparable to 0.31 ± 0.06 mmol/kg dry wt (N = 9) the concentration of PEP found in *Vicia* guard cells (Table II).

DISCUSSION

Malate has been shown to be the major organic anion which accumulates in guard cells during stomatal opening (11). In that study, citrate, glutamate, and aspartate accumulated in an amount equivalent to 63%, 10%, and 8% of malate. The maximum rate of malate accumulation was 110 mmol/kg dry wt·hr (Fig. 4); therefore, an estimate of the required rate of the anaplerotic step would be 200 $mmol/kg_{dry\ weight}$·hr. The carboxylation of PEP is the anaplerotic step in roots when excess cation is taken up (5, 6) and it has been suggested as the anaplerotic step in guard cells (16, 17). The present study provides experimental evidence that PEP carboxylase activity in guard cells is severalfold in excess of that required for the anaplerotic step at the measured PEP concentration and K_{m}PEP (Table II and Fig. 6). The position of this enzyme at a metabolic branch point and the known complex regulation of the enzyme in other systems (*e.g.* 13) suggests a regulatory role for this enzyme in controlling stomatal aperture.

In earlier reports (16, 17), PEP carboxylase activity was greater in epidermal strips than in the remainder of the leaf when expressed on a Chl basis; on a protein basis, activity in epidermal strips was equal to or less than the remainder of the leaf. In one of those studies (16), it was found that PEP carboxylase activity (unit area basis) was 3.4-fold greater in lower epidermal peels than in upper epidermal peels of *Commelina communis*. Stomatal density was 3.8 times greater in the lower epidermal peels. The authors suggested that PEP carboxylase was localized in guard cells. The present results (Fig. 5) show that in lower *Vicia* epidermis, the bulk of activity was in epidermal cells *per se* although the highest specific activity (dry weight basis) was in guard cells (guard cells are only 5% of epidermal volume). Several explanations for this apparent discrepancy may be made. First, it is possible that *Commelina* and *Vicia* have different distributions of PEP carboxylase activity or that upper and lower epidermal cells differ in PEP carboxylase activity. It is also possible that multiple forms of PEP carboxylase exist in C_3 leaves and that the different assay procedures used in these reports preferentially selected for different activities. On the other hand it is possible that the lower epidermis of *Commelina* (with high density of substomatal cavities) can be peeled with less cell disruption and consequent loss of contents.

The distribution of PEP carboxylase activity in the mesophyll tissues confirms a previous study with isolated cells (12) where activity was found to be equal in the two cell types on a protein basis.

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