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Organic acid and potassium accumulation in guard cells during stomatal opening

(palisade parenchyma/spongy parenchyma/epidermis/CO_2 uptake/transpiration)

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ABSTRACT **Leaflets of *Vicia faba* L. with either open or closed stomata were quick-frozen and freeze-dried. Individual guard cell pairs and pure samples of palisade parenchyma, spongy parenchyma, and epidermis lacking guard cells were dissected from the leaflets, weighed, and assayed for organic acids or K^+. K^+ was measured by a new enzymatic method. In guard cells of open stomata, as compared to closed stomata, K^+ was 2- to 4-fold higher, malic acid 6-fold higher, and citric acid 3-fold higher. Both aspartic and glutamic acids were also higher, but the amounts present were low compared to malic and citric acids. Isocitric acid was significantly higher in one experiment, but not in another. Glyceric acid was not increased. Succinic acid was too low to detect by the method used; but in guard cells of open stomata the concentration must have been less than 2% of that of malic acid. Malic acid was higher in the palisade parenchyma from the leaflet with open stomata. The ion balance shows that malic and citric acids provide much of the counter ion for the K^+ taken up during stomatal opening**

Stomata form the interface between leaves and the atmosphere. The opening and closing of these structures is one of the most critical phenomena of plant physiology. Stomata open to permit CO_2 entry and close to prevent H_2O loss. Opening is accomplished by swelling of a pair of guard cells as the result of osmotically generated turgor pressure. It has been known for many years that K^+ concentration in guard cells increases as stomata open (1). More recently, this shuttling of K^+ from epidermal cells to guard cells has been quantified by various techniques [radioactive tracers (2–5), electron microprobe (6, 7), ion-selective microelectrode (8), "rolled" epidermis (9, 10)]. Incoming K^+ accounts for the bulk of the increase in guard cell turgor pressure (7, 9). However, insufficient inorganic anion is cotransported with K^+ to account for electrical neutrality (5, 7, 11–13).

The synthesis of organic anions as a method of balancing cation uptake by plant cells has emerged as an important principle of plant physiology (14). In fact, evidence is now accumulating that K^+ uptake with concomitant synthesis of organic acid is a means of increasing turgor pressure in various types of plant cells (15, 16). It has been repeatedly hypothesized that organic acid synthesis would accompany stomatal opening, but the small dimensions of guard cells and their interspersion with other tissue have discouraged a direct experimental approach to this problem. By using sensitive micromethods we have determined organic acid concentrations in guard cells, epidermal cells, palisade parenchyma cells, and spongy parenchyma cells dissected from frozen-dried leaflets with either open or closed stomata. This paper reports the results.

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MATERIALS AND METHODS

Vicia faba L. plants were grown as described (17). Near the middle of the photoperiod, a leaflet pair was excised under water at the base of the petiole. The leaflets were separated and the base of each placed in water. One leaflet was maintained in darkness; the other was placed in light in a closed container in which the CO_2 tension was decreased by a dish of NaOH. After 1.5 hr the stomatal apertures were estimated microscopically (open stomata averaged 10.4 μm) and the leaflets were frozen in liquid N_2 which had been brought to its freezing point by boiling off part of the liquid under vacuum. (This accelerates freezing by eliminating insulating gas bubbles.) The frozen leaflets were broken into fragments 1–3 mm in diameter (to facilitate drying), dried under vacuum at −35° for 4 days, and stored at −25° (for general histochemical procedures, see ref. 18).

Guard cell pairs and samples of palisade parenchyma, spongy parenchyma, and epidermis lacking guard cells were dissected from the dried leaflets at 18°–20° and 50% relative humidity. Single guard cell pairs and one-to three-cell samples of the other tissues were weighed (3–25 ng) on a quartz fiber ("fishpole") balance (18) and assayed.

Analytical Methods. The amount of material per guard cell pair ranged from 20 fmol of isocitric acid in closed stomata to 5 pmol of K^+ in open stomata. To measure these small quantities, the first analytical steps were carried out in small volumes under oil ("oil well technique", see ref. 18). The enzymatic methods chosen terminated in oxidation or reduction of NAD or NADP. To provide the needed sensitivity, the reduced or oxidized pyridine nucleotide product was then amplified as much as 5000-fold by enzymatic cycling (18). The method given below for glyceric acid illustrates the general style of assay. The methods for aspartic, glutamic, citric, isocitric, and malic acids were used essentially as described (18) except that 1 mM dithiothreitol was included in the specific reagents for isocitric and malic acids and the bovine plasma albumin used in the citric acid method was treated with charcoal to remove the citric acid. (The dithiothreitol was needed for the reason given in the method for glyceric acid.) The K^+ method (unpublished) is based on the requirements of pyruvate kinase for this cation.

Succinic Acid Assay. A method for succinic acid was developed which utilized the principle of Goldberg *et al.* (19). However, in both experiments, the level in guard cells of open stomata proved to be less than 2 mmol/kg (dry). Consequently, succinic acid could not contribute significantly to the total anion pool, and no further effort was made to measure it.

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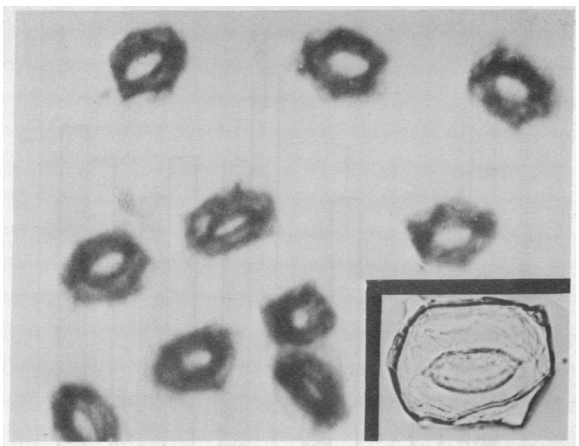

FIG. 1. Frozen-dried guard cell pairs as they appear after dissection. (×265; *Inset*, ×530.)

Whole-Leaf Analyses. $HClO_4$ extracts were prepared from full-thickness portions of fresh or frozen-dried *Vicia* leaflets. These extracts were analyzed directly in the fluorometer with macro versions of the methods used for the micro samples.

Glyceric Acid Assay. The method was based on the oxidation of glyceric acid to hydroxypyruvic acid by NAD^+ and spinach glyceric dehydrogenase (D-glycerate:NAD^+ oxidoreductase, EC 1.1.1.29). The reaction was pulled to completion by destroying the hydroxypyruvic acid with H_2O_2.

Step 1. Each tissue sample was pushed through the oil (in one of 60 wells in an oil well rack) into contact with 0.1 μl of 0.02 M HCl.

Step 2. After heating for 20 min at 80° to destroy endogenous enzymes and NADH, 0.1 μl of reagent was added which consisted of 2 mM NAD^+, glyceric dehydrogenase at 120 μg/ml, 2 mM EDTA, 8 mM H_2O_2, and 2.5 mM dithiothreitol in 100 mM 2-amino-2-methylpropanol (85 mM base:15 mM hydrochloride).

Step 3. After 45 min at 22°–25°, 3 μl of 0.1 M NaOH was added and the rack was heated for 20 min at 80° to destory the excess NAD^+.

Step 4. A 2-μl aliquot of each sample was transferred to a fluorometer tube (in an ice bath) which contained 70 μl of NAD^+ cycling reagent (20) and then was incubated at 20°–25° for 60 min [an amplification of about 2000-fold was provided by pig heart malic dehydrogenase (L-malate:NAD^+ oxidoreductase, EC 1.1.1.37) at 2 μg/ml and yeast alcohol dehydrogenase (alcohol:NAD^+ oxidoreductase, EC 1.1.1.1) at 20 μg/ml in the cycling reagent].

Step 5. After the cycling was stopped with heat (3 min at 95°), the malic acid produced was measured by adding 1 ml of indicator reagent [the malic dehydrogenase–glutamic oxalacetic transaminase reagent of Kato *et al.* (20)], and reading the fluorescence of the NADH formed when oxidation was complete.

For measuring glyceric acid directly in $HClO_4$ extracts of whole leaf, the same reagent was used as in *Step 2* except the concentrations were cut in half and the base-to-acid ratio of the buffer was decreased from 6:1 to 2:1 (pH 10.2) and the volume was increased to 1 ml. A 4-μl sample of $HClO_4$ extract equivalent to 75 μg of dry leaf was used for each assay. The reduction of NAD^+ was measured fluorometrically.

Comment on the specific step of the assay. In direct fluorometric assays (as used with $HClO_4$ extracts), destruction of the reaction product with H_2O_2 is necessary not only to pull the reaction to completion but also because, otherwise, hydroxypyruvic acid forms a highly fluorescent product with NAD^+ [comparable to products formed with acetone or methylethyl ketone (21, 22)]. Use of dithiothreitol and EDTA was necessary to prevent reoxidation of NADH. Dithiothreitol probably acts by reducing tissue disulfides that otherwise could oxidize NADH (possibly by means of a contaminating enzyme such as glutathione reductase in the glyceric dehydrogenase). EDTA probably acts by chelating tissue metals such as Cu^{2+} that catalyze the oxidation of NADH by H_2O_2. The NADH yield from glyceric acid was only 70–80% of theory. This was true both for the hemicalcium salt from Sigma Chemical Co. and for glyceric acid prepared with alkaline phosphatase from enzymatically analyzed 3-phosphaglyceric acid. (Another commercial product gave an even lower yield.) Although glycolic acid reacts with glyceric dehydrogenase (23), the kinetic constants are so unfavorable that less than 0.1% would react under assay conditions. Little or no reaction was obtained with other related compounds (lactic, malic, glyoxylic, citric, isocitric, and *cis*-aconitic acids). Reaction time curves were identical for authentic glyceric acid and for an $HClO_4$ extract of *Vicia* leaflet.

Table 1. Dry mass of single guard cell pairs

Experiments	Mean mass ± SEM, ng (*n*)
1. Light, $-CO_2$	7.1 ± 0.1 (194)
Dark, $+CO_2$	6.6 ± 0.2 (132)
2. Light, $-CO_2$	6.4 ± 0.1 (139)
Dark, $+CO_2$	5.5 ± 0.1 (145)
3. Light, $+CO_2$	5.0 ± 0.2 (10)

Guard cells were hand dissected from frozen-dried leaflets and weighed on a quartz fiber ("fishpole") balance.

RESULTS

Fig. 1 shows the appearance of frozen dried guard cell pairs of open stomata as they were dissected from *Vicia* leaflets. Photographs of single frozen dried palisade parenchyma and spongy parenchyma cells have been published (17).

The mean dry mass of a guard cell pair was 6.1 ng (Table 1). In each of the two experiments shown, guard cells of open stomata had significantly higher dry weight than those of closed stomata. The average difference was 12%.

Whole-Leaf Organic Acids. Absolute levels of organic acids in $HClO_4$ extracts of whole leaf varied from leaf to leaf even when the experimental conditions were presumably similar. However, leaflets from the same leaf had similar acid concentrations. For this reason, the study of stomatal aperture and organic acid concentrations was made exclusively with paired leaflets. The concentration of organic acids in whole leaflet (Fig. 2) was about equal to the weighted average of the concentration in the separate cell types.

Organic Acids in Guard Cells and Three Other Cell Types when Stomata Were Open or Closed. In order to assess changes associated with stomatal opening without possible complications from photosynthesis, opening was induced by light with CO_2 excluded.

Malic acid. The organic acid that showed the most dramatic differences in guard cells between open and closed stomata was malic acid (Fig. 2*A*; Table 2): 5-fold greater in open stomata in one experiment and 6-fold in the other, with an absolute average increase of 77 mmol/kg (dry) (154 meq/kg). Among the other cell types, only palisade parenchyma cells showed a significant difference, being higher by a factor of 2 in the light (Fig. 2*A*; Table 3). It is particularly significant that there was little change in epidermal cells.

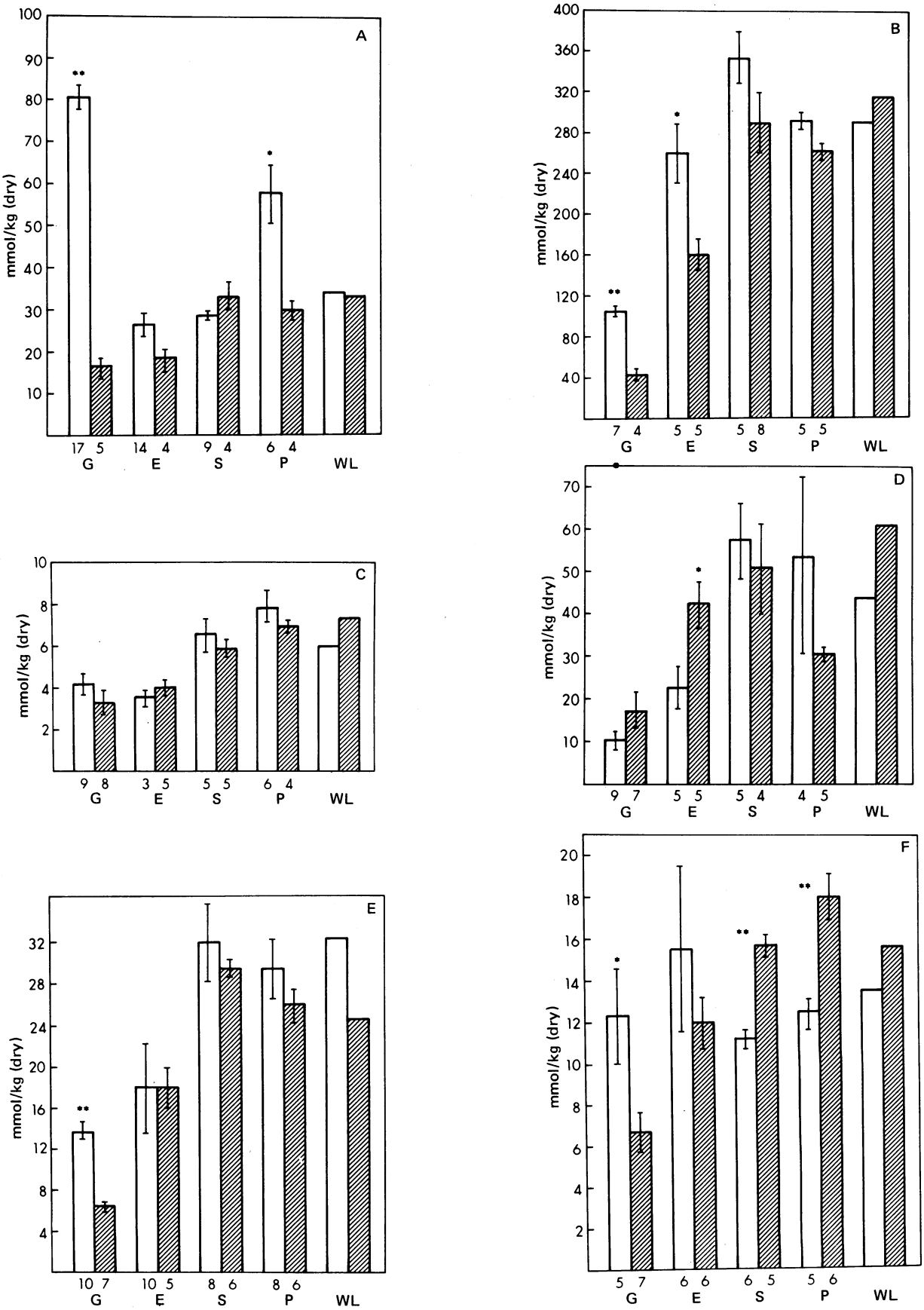

FIG. 2. Organic acid concentrations in four tissues of *Vicia faba* leaflet (G, guard cells; E, epidermal cells; S, spongy cells; P, palisade cells) and in whole leaf (WL). Open bars show averages for a leaflet with open stomata; hatched bars are for the other leaflet (of the same pair) with closed stomata. SEM are indicated by the vertical lines; number of samples is given below each bar; * indicates $P \leq 0.05$; **, $P \leq 0.01$. (*A*) Malic acid; (*B*) citric acid; (*C*) isocitric acid; (*D*) glyceric acid; (*E*) glutamic acid; (*F*) aspartic acid.

Table 2. Effect of status of stomata on K^+ and organic acid concentrations in guard cells of *V. faba*

	Difference, mmol/kg dry weight*		
	Exp. 1	Exp. 2	Av. diff.
Malic acid	65.8 ± 6.9	88.5 ± 6.7	154
Citric acid	61.0 ± 10.8	35.6 ± 10.2	145
Isocitric acid	0.9 ± 0.8	2.9 ± 0.5	6
Glyceric acid	−6.6 ± 4.5	−17.0 ± 6.6	−12
Glutamic acid	7.3 ± 0.6	8.3 ± 1.1	8
Aspartic acid	5.6 ± 2.4	5.4 ± 4.0	6
		Sum	307
Potassium	590 ± 138	473 ± 138	532

* Calculated as open stomata minus closed stomata and shown as mean ± SEM.

Citric acid. Citric acid levels in guard cells of closed stomata were much lower than in other cell types—e.g., 15% of the spongy parenchyma level in experiment 1 and only 5% in experiment 2 (Fig. 2*B*; Tables 2 and 3). However, proportionate changes with light and reduced CO_2 were by far the greatest in guard cells. Citric acid in these cells increased an average of 186% in the two experiments (Fig. 2*B*; Table 2). The absolute increases, although less than for malic acid, amounted to about the same number of acid groups (an average of 145 meq/kg). A relatively smaller increase in the light was observed in epidermal cells; this was significant in one experiment (Fig. 2*B*) but not in the other (Table 3).

Isocitric acid. In experiment 1 there were no significant effects of light on isocitric acid in any cell type (Fig. 2*C*); however, in the second experiment the level of this acid was higher by a factor of 2 in guard cells of open stomata (Table 2). There was poor parallelism between changes in citric acid and in isocitric acid. The equilibrium ratio is about 15:1 (24), whereas the observed ratios varied from 5:1 to 22:1 in guard cells and from 39:1 to 72:1 in the other cell types (in whole leaf, ratios ranged from 33:1 to 48:1). The data suggest that citric acid and isocitric acid were not kept in equilibrium in any cell type.

Glyceric acid. This is the only acid that was lower in guard cells of open stomata than in guard cells of closed stomata (Fig. 2*D*; Table 2). The average absolute *decrease* was only 12 meq/kg, representing less than 10% of the increase in acid equivalents from either malic or citric acids. Glyceric acid was also significantly lower in epidermal cells in the light ($P > 0.02$) but significant changes were not seen in parenchyma cells. The level of glyceric acid was only a third to a sixth as high in guard cells as in epidermal or mesophyll cells. The concentration of glyceric acid in guard cells from a photosynthesizing leaflet was also low (mean ± SEM, 19.6 ± 6.4 mmol/kg, $n = 9$).

Table 3. Malic acid and citric acid in epidermal and parenchyma cells

	Epidermis	Spongy parenchyma	Palisade parenchyma
	Malic acid		
Light	81 ± 22(4)	72 ± 3(3)	144 ± 39(2)
Dark	58 ± 17(4)	64 ± 8(4)	68 ± 8(5)
Diff.	23 ± 28	8 ± 9	76 ± 40
	Citric acid		
Light	133 ± 16(5)	319 ± 36(9)	449 ± 17(5)
Dark	117 ± 16(4)	355 ± 32(6)	378 ± 28(6)
Diff.	16 ± 22	36 ± 48	71 ± 39

The samples are from the same leaflets as experiment 2 of Table 2. Values are mmol/kg dry weight ± SEM for the number of samples in parentheses. None of the changes shown is significant at the $P = 0.05$ level although changes in these acids in the guard cells were highly significant.

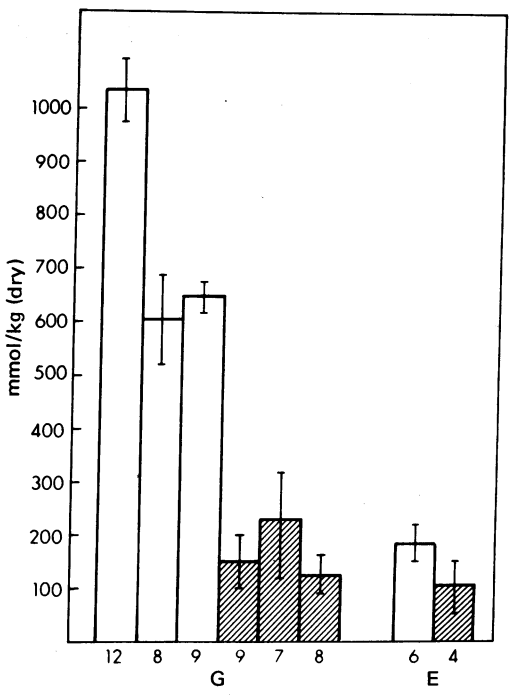

FIG. 3. K^+ concentration in guard cells of *Vicia faba*. Open bars are for a leaflet with open stomata; hatched bars are for a leaflet with closed stomata (the other member of the same pair). To assess variability within each leaflet, samples were taken from three different areas (shown by three separate bars). SEM and numbers of samples are indicated as in Fig. 2.

Glutamic acid. Guard cells of open stomata had twice as much glutamic acid as did those of closed stomata (Fig. 2*E*; Table 2). The average contribution to the acid pool, however, was only 7.8 meq/kg. The other three cell types contained three to five times more glutamic acid than the guard cells of closed stomata, but the levels were not significantly affected by exposure to light.

Aspartic acid. In guard cells the aspartic acid and glutamic acid concentrations were nearly identical in the dark and increased by the same amount in light and reduced CO_2 (Fig. 2*F*; Table 2). In contrast, in other cell types, in the dark aspartic acid was 30–50% lower than glutamic acid. Moreover, light and reduced CO_2 had just the opposite effect on aspartic acid in parenchyma cells to that in guard cells, so that under these conditions aspartic acid levels became nearly equal in all cell types (Fig. 1*F*).

Potassium. In two experiments, K^+ concentration in guard cells increased an average of 532 mmol/kg (Fig. 3; Table 2). In contrast, the concentration of K^+ in epidermal cells was not significantly different under the two experimental conditions and was about the same as in guard cells of closed stomata. To assess the variability within a leaflet, guard cells were dissected from three different areas of each leaflet and assayed for potassium. The scatter was small compared to the difference between leaflets with open and closed stomata.

DISCUSSION

Several methods have been proposed for "isolating" guard cells for analysis by destroying adjacent cells in epidermal strips (9, 25, 26). None of these is completely free of one of two dangers:

contamination with adjacent cells or damage to the guard cells that might produce rapid metabolite changes (27). [The case of citric acid, (Fig. 2C) illustrates the distortion that would result from even modest contamination of guard cells by epidermal or parenchymal cells.] The method used here avoids both these dangers. The only question is whether analysis of such small samples can be sufficiently precise to give reliable answers. The methods themselves have more than the necessary precision (standards in the range of the samples had standard deviations less than 3%). Tissue sample data were more disperse (certainly due in part to biological variation); however, the results clearly demonstrate that variability from all sources can easily be kept within acceptible limits. The validity of the isolated cell data is confirmed by the fact that levels of organic acids measured in acid extracts of the same whole leaflet were close to the weighted average of the isolated cell values for epidermal and mesophyll cells (Fig. 2).

In broad terms, when the stomata opened, each guard cell pair took up 3.2 pmol of K^+ and synthesized or took up 1 peq each of malic and citric acids, leaving 1.2 peq of anion unaccounted for. (Minor increases in glutamic and aspartic acids were partly cancelled out by a decrease in glyceric acid.) Chloride uptake by guard cells has been reported to account for 5, 27, 33, 40, and 73% of K^+ uptake (5, 7, 11–13). The amount of cation that is balanced by organic acid synthesis in vacuolate root sections is dependent upon the relative concentration of chloride in the external medium (28). The same may be true for guard cells. Therefore, it is likely that chloride accounted for the remainder of K^+ uptake.

The data on malic acid are in qualitative agreement with those of Allaway (10). Allaway used his and Hsiao's method (9) of differentially destroying epidermal cells, but not guard cells, in an epidermal strip. After the contents were rinsed from the epidermal cells, the strips were assayed for malic acid and K^+. In those experiments, malic acid balanced 50% of the K^+ taken up. Data on other organic anion concentrations in guard cells *per se* have not been reported. $^{14}CO_2$ incorporations in malic, aspartic, citric, and glutamic acids in epidermal strips (29–31) have been interpreted as evidence that net synthesis of these acids occurs in guard cells upon stomatal opening (13, 29, 30, 32), although the possibility of an exchange reaction was not excluded (33).

The inverse relationships between epidermal glyceric acid concentration and ambient CO_2 level that was observed by Pallas and Wright (34) has been used (35) as evidence that glyceric acid is increased in guard cells of open stomata. However, the present results show that guard cell glyceric acid is *decreased* in open stomata. The decrease in glyceric acid during stomatal opening would be compatible with inhibition of hydrolysis of 3-phosphoglyceric acid as part of a mechanism to convert starch to Krebs cycle acids via the Embden-Meyerhof pathway followed by an anapleurotic step.

The average dry weight of guard cell pairs was greater for open than for closed stomata by 0.5 ng (7%) in one experiment and 0.9 ng (14%) in another. Too much faith should not be placed in these data because of possible systematic dissection errors. Nevertheless, the possibility of an increase of this magnitude should not be dismissed. If all of the organic acid increments were imported, and the missing anion were chloride, the sum of the K^+, organic acid, and chloride increments would be 0.29 ng per guard cell pair. It is more likely that organic acids were generated in the guard cells, which would decrease the calculated increase to 0.17 ng. On the other hand, it seems possible that other soluble components might be carried in with the influx of water to give a dry weight increase as large as that observed.

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