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Potassium Chloride as Stomatal Osmoticum in *Allium cepa* L., a Species Devoid of Starch in Guard Cells[1]

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HEIDE SCHNABL[2] AND KLAUS RASCHKE[3]
MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

K^+ and Cl^- contents of guard cells and of ordinary epidermal cells were determined in epidermal samples of *Allium cepa* L. by electron probe microanalysis; malate contents of the same samples were determined by enzymic oxidation. KCl was, in general, the major osmoticum in guard cells, irrespective of whether stomata had opened on leaves or in epidermal strips floating on solutions. The solute requirement varied between 50 and 110 femtomoles KCl per micrometer increase in aperture per pair of guard cells. Stomata did not open on solutions of K iminodiacetate, presumably because its anion could not be taken up. Stomata opened if KCl or KBr was provided. Taken together, the results indicate that the absence of starch from guard cells deprived them of the ability to produce malate in amounts of osmotic consequence and that the presence of absorbable Cl^- (or Br^-) was necessary for stomatal opening.

Previous nutrient supply of the plants determined whether the charges of K^+ in guard cells were completely balanced by Cl^- or only partially. Addition of K_2SO_4 to the nutrient solution reduced the participation of Cl^- in stomatal ion transfer, even if epidermal strips of these plants were later exposed to KCl solution. The anion supplying the charge complement in these cases is not known.

Although malate appeared not to participate in stomatal ion transfer in onion, epidermal samples of this species did contain malate. Malate accumulated in the epidermis of leaves put into the light but disappeared from illuminated epidermal strips floating on solutions. In whole leaves, epidermal malate content was positively correlated with stomatal opening; in epidermal strips floating on solutions, the correlation was negative or absent.

Stomatal opening is the osmotic effect of an accumulation of salts of K^+, and sometimes Na^+, in guard cells; chloride and malate are the most important anions (for review see ref. 15).[4] Alkali and chloride ions are taken up by guard cells from their environment; malate can be made within these cells by carboxylation of P-enolpyruvate (14). P-enolpyruvate is very likely derived from starch (4). In guard cells of *Vicia faba*, the balance between potassium chloride and potassium malate was determined by the availability of Cl^- (17).

Guard cells of *V. faba* contain chloroplasts filled with starch, but guard cells of *Allium* species possess only poorly developed chloroplasts (1, 10, 22, 23) which do not contain starch (1, 2, 8, 22, 24) or only traces of it (11). Guard cells of *Allium* species, therefore, appear to lack substrate for the production of the quantities of malate required for osmotic purposes. Possibly, these cells satisfy their anion requirement solely through import of Cl^-. Indeed, there are indications for a lack of involvement of malate metabolism in the stomatal movement of onion: epidermal strips of *V. faba* possess phosphorylase activity, those of *Allium cepa* do not (13, 18, 22). Aluminum ions inhibit stomatal opening in *V. faba* and other species containing starch in guard cells (21) but not in *A. cepa* (22). This difference in stomatal sensitivity to Al^{3+} has been ascribed to the inhibition of starch breakdown and acceleration of starch synthesis by precipitation of Pi as $AlPO_4$ (21). Phosphorylase activity of isolated epidermis of *V. faba* could be stopped by Al^{3+} *in vitro* (18). Also, guard cells of *Allium* species cannot make sufficient malate from sources other than starch (like mucilage; 19). On the other hand, good evidence exists for an involvement of Cl^-. Schnabl and Ziegler (22) subjected onion epidermis to histochemical and electron microprobe analyses. Their results led to the suggestion that onion guard cells contain K^+ and Cl^- in equivalent amounts. We set out to test this contention. We calibrated an electron probe microanalyzer for the simultaneous determination of K and Cl in epidermal cells, including guard cells (9, 17). After K and Cl contents had been determined in epidermal samples, we measured the malate content of the same material. If the malate content changed with stomatal opening in positive correlation and in osmotically significant amounts, as it did in *Commelina communis* and *V. faba* (25, 26), an osmotic function of malate would be indicated. An absolute requirement of Cl^- for stomatal functioning in onion would then appear unlikely. We added a further aspect to our investigation. It appears possible that availability of Cl^- is essential for stomatal opening in onion. If this is true, modification of Cl^- nutrition may cause changes in stomatal ion contents and stomatal behavior. We therefore compared plant material that had received Cl^- in trace amounts only (Hoagland solution, 3 μeq Cl^-/l) with material whose nutrition was supplemented with KCl or K_2SO_4, the latter as a further control.

MATERIALS AND METHODS

Plants. Plants of *A. cepa* (cv. Stuttgarter, Joseph Harris Co. Rochester, N.Y.) were grown for 8 to 12 days from sets in soil consisting of two parts Bacto potting soil (Michigan Peat, Houston, Tex.) and one part Perlite (W. R. Grace & Co.) in a growth chamber. All plants were watered three times per week, once with full strength Hoagland solution (pH 6.5), the other two times with

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[2] Permanent address: Institut für Botanik und Mikrobiologie der Technischen, Universität München, Arcisstrasse 21, 8000 München 2, Federal Republic of Germany.

[3] Present address: Pflanzenphysiologisches Institut der Universität Göttingen, Untere Karspüle 2, 3400 Göttingen, Federal Republic of Germany.

[4] Nelson and Mayo (12) recently suggested that K^+ is not involved as a major osmoticum in guard cells of *Paphiopedilum leeanum*. However, these authors did not verify that stomata opened during their experiment. One of us (K.R.), using microscope and gas analyzers, found that stomata of *P. leeanum* open only slightly in the light and in CO_2-free air and that small changes in the K^+ content of guard cells did occur in correlation with the observed changes in stomatal aperture.

distilled H_2O. Two groups of plants received supplements of 5 mM of either KCl or K_2SO_4. All plants were grown at a daylength of 16 h (85 w/m^2 from fluorescent tubes). The air temperature was 22 C during the light period and 20 C during the dark period. RH was about 85%, day and night.

Stomatal Opening in Illuminated Leaves. Pots with onion plants were taken from the growth chamber during the dark period and placed under mercury vapor lamps (General Electric H 400 RDX 33-1 behind a 5-cm-deep water filter). The irradiance was 85 w/m^2, air temperature 21 C. Epidermal strips were sampled before exposure of the plants to the light, after 20 and after 60 min in the light, and then analyzed as described below.

Preparation and Incubation of Epidermal Samples. Leaves (about 10 cm long) were cut in the morning before the beginning of the light period and divided into sections (about 2 cm long) which were floated on distilled H_2O in the dark. Samples of the lower epidermis were peeled from these sections in room light (from fluorescent tubes) and rubbed on their mesophyll side with a dissecting needle in distilled H_2O in order to remove any adhering mesophyll cells. The strips were then rinsed and collected on deionized H_2O. For the determination of the initial conditions, some samples were immediately extracted for malate analysis or prepared for electron probe microanalysis after measurement of stomatal apertures. Other epidermal samples were transferred to small plastic dishes containing the incubation media. The dishes were placed under inverted crystallization dishes (150-mm diameter, 75-mm height) in the light (85 w/m^2). A stream of humidified air was passed through the crystallization dishes at 50 liters/h. The air was either free of CO_2 or contained about 320 μl CO_2/l. Epidermal samples were incubated in the light at 21 C for 4 h. The incubation media were buffered with Mes (10 mM, pH 5.6) and contained 0.09 to 0.11 eq K^+/l and 0 to 0.1 eq Cl^-/l. Iminodiacetate (J. T. Baker Chemical Co.) was used to balance K^+ not balanced by Cl^-. Indirect evidence indicates that guard cells do not absorb iminodiacetate (16, 17). The solutions were prepared with double distilled H_2O. The estimated maximal concentration of Cl^- resulting from impurities in the chemicals was 10^{-6} eq/l. No Cl^- could be detected in water in contact with plastic dishes for 24 h (17).

After incubation, the area of the epidermal strips was measured with calipers and the width of the stomatal apertures under a microscope, using a calibrated ocular micrometer. The strips measured from 3 to 5 mm × 6 to 8 mm. Ten stomatal apertures were determined on each strip and each sample consisted on the average of five epidermal strips. The average stomatal density was 120 mm^{-2}. Following these measurements, the epidermal strips were either extracted or prepared for microprobe analysis.

Electron-probe Microanalysis. An Applied Research Laboratories (Sunland, Calif.) model EMX-SM microanalyzer was calibrated at an acceleration voltage of 15 kev with small crystals of KCl, KBr, and K-oxalate of known volumes (9). The current on the slide was 22 namp. Samples of calibration curves have been given in reference 17. Epidermal strips were prepared for the microprobe by washing in ice-cold 50 mM $Ca(NO_3)_2$ followed by 0.1 mM $Ca(NO_3)_2$ (9). The strips were then placed on aluminum slides, blotted, rapidly frozen in liquid N_2 and freeze-dried at −40 C (9). The desiccated samples were fixed to aluminum slides by means of Scotch No. 400 double-coated tape (The 3-M Company, St. Paul, Minn.) and then coated with carbon. The x-ray fluorescence emitted by single pairs of guard cells was analyzed by scanning areas measuring 40 × 50 μm^2 with the electron beam. For each stoma an equal area of adjoining ordinary epidermal cells was scanned and its emission determined. Background radiation was determined by counting scintillations when the x-ray spectrometers were set off peak. Each epidermal sample consisted of 5 to 8 epidermal strips, and measurements were made on 10 stomata and 10 epidermal areas free of guard cells in each strip. The results are thus based on at least 50 measurements per treatment. Element contents were determined for each 40- × 50-μm^2 field individually by subtraction of the off-peak counts from the number of scintillations and then referring to the calibration curves.

Table I. *Stomatal Apertures and Malate Contents of Isolated Epidermal Strips of A. cepa Incubated in the Presence of Various Amounts of Cl^- for 4 h at 21 C*

The balancing anion was iminodiacetate. Mean ± 1 SD.

Treatment	Cl^- Content	Stomatal Aperture	Malate Content	
	eq/l	*μm*	*pmol/mm^2*	*feq/stoma*
Initial condition		4.7 ± 1.5	73.5 ± 11.2	1200 ± 180
Double distilled H_2O				
$-CO_2$, light	0	4.4 ± 0.8	12.6 ± 1.6	210 ± 30
$-CO_2$, dark	0	4.2 ± 0.7	10.9 ± 2.2	180 ± 40
$+CO_2$[a], light	0	4.4 ± 0.5	8.9 ± 1.7	150 ± 30
$+CO_2$, dark	0	4.5 ± 0.7	12.8 ± 2.1	210 ± 30
10^{-1} eq K^+/				
$-CO_2$, light	10^{-1}	5.6 ± 0.5	9.5 ± 2.4	160 ± 40
	10^{-2}	2.3 ± 0.1	11.1 ± 2.1	180 ± 30
	10^{-3}	0.7 ± 0.05	13.6 ± 2.3	220 ± 40
	10^{-4}	0.7 ± 0.04	11.6 ± 3.6	190 ± 60
	10^{-5}	0.7 ± 0.05	12.7 ± 3.5	210 ± 60
	0	0.0	10.3 ± 2.3	170 ± 40

[a] About 320 μl CO_2/l.

Determination of Malate. Fresh or freeze-dried epidermal samples (at 5 strips each) were extracted three times with 5 ml of boiling 80% alkaline ethanol for 20 min each time. The extracts were joined and analyzed for malate, as described before, by enzymic oxidation of malate coupled to the reduction of NAD (7, 17, 26). It was established earlier that the malate content of epidermal samples did not decline during freeze-drying and microprobe analysis (17). Epidermal strips which had been under the electron beam could therefore be used for the determination of malate. Amounts of malate were expressed in mol/mm^2 or converted to eq/stoma by division by the average stomatal frequency (120 mm^{-2}) and multiplication by two, on the assumption that the pH in the vacuoles of guard cells of open stomata was sufficiently above the pK_2 of malic acid (= 5.1).

Statistics. Levels of significance were determined by application of the *t* test.

RESULTS

Epidermal Malate Content and Stomatal Aperture as Depending on the Availability of Cl^-; Epidermis Floating on Solutions. Earlier investigations into stomatal behavior and ion contents of isolated epidermal tissue of *V. faba* showed that in the absence of Cl^-, stomatal opening and malate accumulation were positively correlated with each other. Malate accumulation decreased with increasing additions of Cl^- to the incubation medium (17, 26). No such correlations appeared in isolated epidermal tissue of *A. cepa* (Table I). The malate content of onion epidermis was highest (about 74 pmol/mm^2) when samples were taken from the leaves, and it declined during incubation to about 12 pmol/mm^2, irrespective of the treatment, whether stomata closed or opened, whether Cl^- was present in the solution or not, whether CO_2 was present or not, and whether there was light or darkness.

In epidermal strips floating on distilled H_2O, stomata stayed open over a period of 4 h. They closed if the incubation medium contained K^+ in combination with the presumably nonabsorbable anion iminodiacetate.[5] Closing could be reduced, prevented, or overcome by addition of increasing amounts of Cl^- to the medium.

[5] Abbreviation: KIDA: potassium iminodiacetate.

Table II. *Balance between K^+ and Cl^- in Pairs of Guard Cells and Malate Contents of Epidermal Samples Taken from Leaves of A. cepa in Darkness and after an Exposure to Light (85 w/m²) for 20 Min and 60 Min at 21 C*

Means ± SD.[a]

Nutrient Supplement[b]	Treatment	Stomatal Aperture	K^+ Content	Cl^- Content	Cl^-/K^+[c]	Malate Content
		μm	*feq/stoma*		*eq/eq*	*feq/stoma*
None	Dark	2.6 ± 1.0	580 ± 20	490 ± 180	0.85 ± 0.55	700 ± 80
	20 min light	3.6 ± 1.3	680 ± 230	460 ± 170	0.51 ± 0.40	1300 ± 100
	60 min light	5.5 ± 0.8	730 ± 130	600 ± 130	0.90 ± 0.28	1300 ± 100
5 mM KCl	Dark	3.6 ± 2.8	540 ± 200	610 ± 200	1.10 ± 0.40	360 ± 60
	20 min light	6.4 ± 2.3	640 ± 160	650 ± 230	1.20 ± 0.80	1000 ± 140
	60 min light	8.7 ± 1.3	840 ± 200	880 ± 180	1.14 ± 0.46	1240 ± 170
5 mM K_2SO_4	Dark	2.3 ± 0.7	100 ± 10	450 ± 90	4.00 ± 1.20	670 ± 50
	20 min light	5.2 ± 0.6	560 ± 270	490 ± 160	0.92 ± 0.70	900 ± 150
	60 min light	8.8 ± 1.3	620 ± 300	500 ± 140	0.83 ± 0.70	1200 ± 100
Increases during exposure to light						
Nutrient Supplement	Time in Light	Δ Aperture	ΔK^+	ΔCl^-	$\Delta Cl^-/\Delta K^+$	Δ Malate
	min	*μm*	*feq/stoma*		*eq/eq*	*feq/stoma*
None	60	2.9	150	110	0.73	600
5 mM KCl	60	5.1	300	270	0.90	880
5 mM K_2SO_4	60	6.5	520	50	0.10	530

[a] All increases in stomatal aperture and K^+ and Cl^- contents that occurred during the 60-min exposure to light are significant at $P < 0.001$ except the change in Cl^- content in the K_2SO_4 material.

[b] Supplement of Hoagland solution during cultivation of plants.

[c] Means of individual Cl^-/K^+ ratios, not ratio of mean contents of K^+ and Cl^-; values in this column are therefore not equal to the ratio of values in the two columns on K^+ and Cl^- contents.

A Cl^- concentration as high as 0.1 eq/l was required to cause stomata to open beyond their initial aperture. Apparently, availability of Cl^- is essential not only for the inflation of guard cells of onion but also for the maintenance of inflation. A good supply of Cl^- may therefore be necessary for stomatal functioning in onion plants.

K^+, Cl^-, and Malate in Epidermis from Illuminated Plants.[6] Three sets of plants were grown to see whether improved supply with Cl^- affects stomatal activity: one at a low level of Cl^-, using Hoagland solution, which contains 3 μeq Cl^-/l and 5 meq K^+/l (3); one with a supplement of 5 mM KCl to the Hoagland solution, resulting in 5 meq Cl^-/l and 10 meq K^+/l, and a third set which received Hoagland solution with a supplement of 5 mM K_2SO_4, resulting in 3 μeq Cl^-/l and 15 meq K^+/l. The third set was included as additional control; a higher K^+ concentration than in the KCl treatment was chosen because roots absorb K^+ from solutions of K_2SO_4 at a slower rate than from those of KCl (5). Pretreatment of plants with KCl or K_2SO_4 led to faster and wider stomatal opening than in plants that had received straight Hoagland solution (Table II). Yet in all treatments, K^+ contents of guard cells of open stomata were balanced by approximately equivalent amounts of Cl^-. Although Cl^-/K^+ ratios were > 1 in the pretreatments with KCl, they were not significantly different from 1.0. A test was made to see whether differences between equivalents of K^+ and Cl^- in guard cells were significantly different from zero. This was not the case, except in the 60-min light treatment of control plants, where K^+ exceeded Cl^- by 110 feq/stoma at $P = 0.02$, and in guard cells from darkened leaves from K_2SO_4 plants, where Cl^- exceeded K^+ by as much as 380 feq/stoma at $P < 0.001$.

The lower portion of Table II shows that guard cells imported about equivalent amounts of K^+ and Cl^- when stomata opened in control plants and in plants that were pretreated with KCl. Guard cells of K_2SO_4 plants imported much less Cl^- than K^+; their $\Delta Cl^-/\Delta K^+$ ratio was as low as 0.1. Supplementation of the Hoagland solution with KCl or K_2SO_4 did not obviously affect the K^+ and Cl^- contents of the ordinary epidermal cells (see Table IV, A). In all treatments, they contained more Cl^- than K^+. This preponderance of Cl^- found no correspondence in the guard cells (Table II).

The malate content of the epidermis (including the guard cells) increased during stomatal opening (Table IV, A). These increases were not paralleled by increases in K^+ in the ordinary epidermal cells. If all epidermal malate was concentrated in the guard cells it could not have been balanced by K^+ either, particularly not after 60 min of illumination, because all of the K^+ found in guard cells would have been required to balance Cl^-. However, in material pretreated with K_2SO_4, malate could have participated in stomatal movement; the increase in epidermal malate is of the same order of magnitude as the deficiency in stomatal chloride (lower portion of Table II).

K^+, Cl^-, and Malate in Epidermis Incubated with Solutions. Epidermal strips with closed stomata were taken from plants of each nutritional pretreatment and incubated with 0.1 eq K^+/l in the absence or presence of 0.1 eq Cl^-/l. As in the first experiment (Table I), stomatal aperture, and K^+ and Cl^- contents of guard cells declined when no Cl^- was in the medium; aperture in the KCl pretreatment was an exception (Table III). All exposures to KCl led to increases in the guard cell contents of K^+ and Cl^- and to stomatal opening. Increases in K^+ and Cl^- balanced each other in control and KCl material but not in guard cells from K_2SO_4 plants. The $\Delta Cl^-/\Delta K^+$ ratios (lower portion of Table III) of 0.95 (control) and 1.62 (KCl) are not significantly different from 1.00, but the value of 0.32 (for K_2SO_4) is. Differences between the numbers of K^+ and Cl^- equivalents in guard cells were also tested for significance. They did not deviate from zero except in the following samples: KCl treatment of unsupplemented material: K^+ exceeded Cl^- by 250 feq/stoma at $P < 0.001$; KIDA treatment of K_2SO_4 material: Cl^- exceeded K^+ by 360 feq/stoma at $P < 0.001$; KCl treatment of K_2SO_4 material: Cl^- exceeded K^+ by 170 feq/stoma at $P = 0.02$.

The malate content of isolated epidermal samples declined during incubation (Tables III and IV). As in epidermis analyzed

[6] Although electron probe microanalysis does not distinguish between K and K^+ on one hand and Cl and Cl^- on the other, we assume that virtually all K and Cl was present in salts; therefore, we use K^+ and Cl^- throughout this paper.

Table III. *Balance between K^+ and Cl^- in Pairs of Guard Cells and Malate Contents of Epidermal Samples of A. cepa after Incubation with 0.1 M K Iminodiacetate (KIDA) or KCl for 4 h in CO_2-free Air at 21 C and an Irradiance of 85 w/m^2.*

Solutions were buffered at pH 5.6 with 10 mM Mes. Means ± 1 SD.[a]

Nutrient Supplement[b]	Treatment	Stomatal Aperture	K^+ Content	Cl^- Content	Cl^-/K^+[c]	Malate Content
		μm	*feq/stoma*		*eq/eq*	*feq/stoma*
None	Initial condition	4.7 ± 1.5	450 ± 170	220 ± 50	0.6 ± 0.3	1200 ± 130
	KIDA	2.1 ± 0.2	200 ± 90	180 ± 50	1.0 ± 0.2	410 ± 100
	KCl	8.7 ± 1.9	890 ± 230	640 ± 120	0.9 ± 0.3	460 ± 90
5 mM KCl	Initial condition	5.9 ± 4.2	680 ± 170	640 ± 280	1.0 ± 0.4	1310 ± 300
	KIDA	6.8 ± 3.6[d]	570 ± 420[d]	480 ± 250	1.8 ± 1.5[g]	1100 ± 170
	KCl	8.9 ± 3.2	920 ± 250	1030 ± 310	1.3 ± 0.5[e]	470 ± 110
5 mM K_2SO_4	Initial condition	2.7 ± 1.1	250 ± 100	570 ± 210	2.5 ± 1.2	1250 ± 200
	KIDA	1.8 ± 1.6	190 ± 50	550 ± 180[d]	3.5 ± 1.5	580 ± 40
	KCl	3.9 ± 2.5	470 ± 230	640 ± 200[f]	1.9 ± 1.2	500 ± 60

Changes that occurred during incubation

Nutrient Supplement[b]	Treatment	Δ Aperture	ΔK^+	ΔCl^-	$\Delta Cl^-/\Delta K^+$	Δ Malate
		μm	*feq/stoma*		*eq/eq*	*feq/stoma*
None	KIDA	−2.6	−250	−40	0.16	−990
	KCl	4.0	440	420	0.95	−740
5 mM KCl	KIDA	0.9[d]	−110[d]	−160	1.45	−210
	KCl	3.0	240	390	1.62	−840
5 mM K_2SO_4	KIDA	−0.9	−60	−20[d]	0.33	−670
	KCl	1.2	222	70	0.32	−750

[a] Differences in stomatal aperture and contents of K^+ and Cl^- are significant at $P < 0.005$ within each pretreatment ("nutrient supplement"), unless indicated otherwise.

[b, c] See corresponding footnotes to Table II.

[d] Difference with initial condition or change not significant.

[e] Difference with initial condition significant at $P = 0.05$.

[f] Difference with initial condition significant at $P = 0.025$.

[g] Difference with initial condition significant at $P = 0.005$.

Table IV. *Contents of K^+, Cl^- of the Ordinary Epidermal Cells, and Malate Content of Epidermal Tissue of A. cepa*

K^+ and Cl^- were measured by scanning a rectangle of 40 × 50 μm^2 and avoiding cell walls (identical with the area scanned for the measurement of the element content of one stomatal apparatus). "A" belongs to the experiment of Table II; "B" belongs to the experiment of Table III.

Nutrient Supplement	Treatment	K^+ Content	Cl^- Content	Cl^-/K^+	Malate
		peq/mm^2		*eq/eq*	*peq/mm^2*
A. Sampled from leaves					
None	Dark	85 ± 60	225 ± 60	2.6	85 ± 20
	20 min light	55 ± 25	170 ± 70	3.1	159 ± 24
	60 min light	65 ± 35	140 ± 35	2.2	159 ± 26
KCl	Dark	45 ± 40	150 ± 40	3.2	44 ± 8
	20 min light	25 ± 5	200 ± 45	8.0	123 ± 18
	60 min light	30 ± 25	175 ± 65	5.8	152 ± 21
K_2SO_4	Dark	35 ± 15	200 ± 30	5.7	82 ± 13
	20 min light	50 ± 10	150 ± 25	3.0	110 ± 19
B. Incubation on solutions					
None	Initial	60 ± 30	85 ± 35	1.4	148 ± 16
	KIDA	85 ± 50	90 ± 30	1.1	50 ± 12
	KCl	105 ± 50	115 ± 40	1.1	57 ± 12
KCl	Initial	90 ± 45	225 ± 50	2.5	160 ± 69
	KIDA	85 ± 60	220 ± 45	2.6	136 ± 42
	KCl	40 ± 30	175 ± 40	4.4	57 ± 13
K_2SO_4	KCl	85 ± 50	200 ± 50	2.4	62 ± 7

immediately after removal from the leaf, the combined equivalents of Cl^- and malate in incubated epidermis by far exceeded the equivalents of K^+. After incubation on solutions, the Cl^-/K^+ ratio of the ordinary epidermal cells was about 1 in epidermis from control plants and > 1 in epidermis from plants that had received supplements of KCl or K_2SO_4 (Table IV, B).

Substitution of Cl^- by other Halogenides. Epidermal strips were floated on buffered 0.1 M solutions of KF, KCl, KBr, and KI. As expected, KF produced complete stomatal closure. KBr was as effective as KCl in keeping stomata open (Table V), and KI reduced stomatal aperture. The contents of the strips exposed to KCl and KBr were analyzed and had very similar compositions except that in KBr treatment Cl^- and Br^- contributed about equally to the anion content of the guard cells. Of the anions gained during incubation virtually all were Br^- (lower portion of Table V).

Table V. *Uptake of Br^- or Cl^- by Guard Cells in Isolated Epidermal Strips of A. cepa during Incubation with 0.1 M KBr or KCl for 4 h in CO_2-free Air*

Conditions including buffering, as for Table IV. Plants did not receive a nutrient supplement during cultivation. Means ± 1 SD.

Treatment	Aperture	K^+ Content	Cl^- Content	Br^- Content	Cl^-/K^+	Br^-/K^+	Malate Content	
	μm	*feq/stoma*			*eq/eq*		*peq/mm²*	*feq/stoma*
Initial condition	3.4 ± 0.9	450 ± 140	370 ± 60		0.82 ± 0.20		145 ± 11	1200 ± 90
KCl	4.9 ± 2.8	690 ± 200	800 ± 200		1.27 ± 0.52		50 ± 11	410 ± 95
KBr	4.6 ± 3.2	640 ± 200	380 ± 150	420 ± 210	0.59 ± 0.15	0.65 ± 0.21	53 ± 10	430 ± 80
Changes that occurred during incubation								
Treatment	Δ Aperture	ΔK^+	ΔCl^-	ΔBr^-	$\Delta Cl^-/\Delta K^+$	$\Delta Br^-/\Delta K^+$	Δ Malate	
	μm	*feq/stoma*			*eq/eq*		*feq/stoma*	
KCl	1.5	240	430		1.79		−790	
KBr	1.2	190	10	420	0.05	2.21	−770	

Table VI. *Comparison of Ion Requirements for Stomatal Opening in A. cepa and V. faba*

(Increases in stomatal contents of K^+ and Cl^- and in epidermal contents of malate divided by the observed changes in stomatal aperture.) All plants were grown with Hoagland solution as nutrient source, supplemented as stated.

Stomata Opened	Reference	Species	Nutrient Supplement	Treatment (Time in Light)		In Guard Cells		In Epidermis
						ΔK^+	ΔCl^-	Δ Malate
						feq/μm·stoma		
On plants	Table III	*A. cepa*	None	1 h		52	40	207
			KCl			59	53	173
			K_2SO_4			80	8	82
	9, 26	*V. faba*	None	3 or 4 h		404	22	~1000[a]
In isolated epidermis	Table IV	*A. cepa*	None	4 h	KIDA		Stomatal closure	
					KCl	110	105	−185
			KCl		KIDA	−122	−178	−233
					KCl	80	130	−280
			K_2SO_4		KIDA		Stomatal closure	
					KCl	74	23	−625
	17	*V. faba*	None	4 h	KIDA	433	26	396
					KCl	188	85	93

[a] Electron microprobe and malate analyses were performed on separate materials.

DISCUSSION

In general, guard cells of *A. cepa* imported roughly equivalent amounts of K^+ and Cl^- when stomata opened in illuminated leaves of intact plants or in isolated epidermal strips floating on solutions (Tables II and III); Br^- could substitute for Cl^- (Table V). If Cl^- was absent from the solutions, stomata were not able to open (Table III, KIDA treatment). These results support Schnabl and Ziegler's (22) contention that guard cells of onion (and probably other *Allium* species) import K^+ and Cl^- during stomatal opening in equivalent amounts. The equivalence of K^+ and Cl^- was rarely exact, insofar as the scatter of our data allows a statement on exactness. Pretreatment of plants with KCl increased the ratio Cl^-/K^+ in guard cells (Table II). Pretreatment with K_2SO_4 led to an unexpected reduction of the participation of Cl^- in stomatal movements: only one-tenth of the increase in stomatal K^+ was balanced by Cl^- (Table II, $\Delta Cl^-/\Delta K^+$). The reduced participation of Cl^- in stomatal ion transfer of K_2SO_4 plants appeared even on epidermal samples that had been incubated with KCl for 4 h and contained absolute amounts of Cl^- that exceeded those of K^+ (Table III, Cl^-/K^+ and $\Delta Cl^-/\Delta K^+$). We cannot explain the discrepancy between the presence of Cl^- in the tissue (Table IV) and the failure of the guard cells to use it as a counter-ion for K^+.

Onion epidermis contained malate and changes in the malate level occurred when stomata opened (Tables II, III, and IV). These changes were positively correlated with stomatal opening when it occurred on leaves (Tables II and IV A) but were negatively correlated when stomata opened in epidermal strips floating on solutions (Tables II, III, and IV). Apparently the epidermis imported malate (or a malate precursor) from the photosynthesizing mesophyll in the first case and lost malate to the solutions in the second. Loss of malate from the tissue was never complete, supporting the notion that the presence of some malate in the epidermis may be essential for stomatal functioning in onion plants (20).

In epidermal strips placed on distilled H_2O, stomata stayed open; they closed if the water contained KIDA (Table I). Closing could be partially or fully prevented by substituting Cl^- for IDA^- in increasing amounts. Table III shows that guard cells lost K^+ and Cl^- when in contact with a 0.1 M solution of KIDA but gained K^+ and Cl^- when exposed to 0.1 M KCl. We interpret these observations as resulting from a combination of a relatively high salt permeability of guard cell membranes with a high charge density of the pectinaceous guard cell walls (15). R. A. Saftner and K. Raschke (unpublished) measured Donnan potentials of guard cell walls of various species, including onion; in 30 mM KCl (pH 7), the wall potential was about −65 mv. We conclude that the density of negative charges in the walls is > 0.3 M and consider it possible that in epidermal strips floating on distilled H_2O, Donnan exclusion of anions from walls (and membranes?) could effectively prevent salt leakage from guard cells. If KIDA were added in increasing amounts, increasing numbers of fixed negative charges would be neutralized by K^+ and leakage of Cl^- (in association with K^+) would become possible unless Cl^- were added to the external solution to reduce or even invert the activity

gradient of Cl^-, allowing stomata to gain Cl^- and open. In this context early reports on the production of very wide stomatal opening in paradermal sections or epidermal strips of *Allium* species are of interest: Arends (2) observed apertures "as wide as on leaves kept under optimal conditions" in sections brought into solutions of 0.5 M NaCl, and Fujino (6) measured apertures as wide as 11.8 μm in epidermal strips floating on 0.5 M KCl.

For a summary, we return to the suggestion that the presence of starch in guard cells is a requirement for malate production during import of K^+ and that absence of starch introduces a requirement of Cl^- for stomatal functioning. In Table VI ion contents of guard cells of *A. cepa*, a species devoid of starch in guard cells, are juxtaposed with those of *V. faba*, a species possessing starch in guard cells. The data on *V. faba* are from earlier investigations conducted in this laboratory (9, 18, 26); the same methods were used as in the present investigation. To facilitate comparison, changes in ion contents were related to the observed simultaneous changes in stomatal aperture. In both *A. cepa* and *V. faba*, the amount of K^+ imported into a pair of guard cells per μm increase in aperture varied by a factor of about 2 (from 52 to 110 feq/μm·stoma in *A. cepa* and from 188 to 433 feq/μm·stoma in *V. faba*). Values obtained from epidermis sampled from illuminated plants roughly agreed with those obtained from isolated epidermis incubated on solutions. However, per μm increase in stomatal opening, stomata of *A. cepa* imported less K^+ than those of *V. faba*. A difference between the two species in the elastic modulus of the guard cell walls may have been the cause. Stomata of *A. cepa* are also smaller than those of *V. faba*, which may affect the ratio between changes in aperture and changes in guard cell volume.

The relationship between anions and cations in guard cells shows that in *A. cepa* all K^+ can be balanced by Cl^-. In K_2SO_4-treated plants another anion in addition to Cl^- must have been present in guard cells.

In isolated epidermis exposed to Cl^--free solutions (KIDA treatments), stomata of *V. faba* and *A. cepa* differed in their behavior. While the starch-free guard cells of *A. cepa* could not swell and instead lost solutes, stomata of *V. faba* opened as a consequence of production of malate and accumulation of K^+. Although the malate content of onion epidermis declined during incubation, there was still sufficient malate present that could have been imported by the guard cells for stomatal opening but was not (Table III, upper portion). When malate is required for stomatal opening it apparently has to be produced within guard cells and the presence of starch is essential for it in these cells.

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