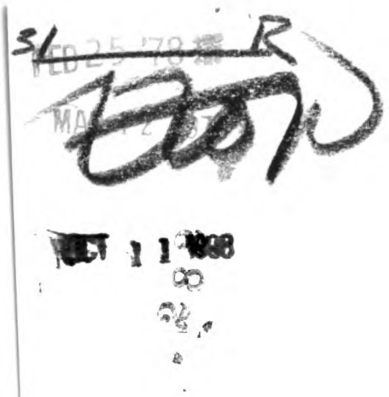
MALATE CONTENT OF LEAF EPIDERMIS IN RELATION TO STOMATAL APERTURE AS EFFECTED BY EXOGENOUS CHLORIDE

Thesis for the Degree of M. S.

MICHIGAN STATE UNIVERSITY

CAROL ANNE VAN KIRK

1977



ABSTRACT

MALATE CONTENT OF LEAF EPIDERMIS
IN RELATION TO STOMATAL APERTURE AS
EFFECTED BY EXOGENOUS CHLORIDE

By

Carol Anne Van Kirk

Stomatal aperture is determined by the osmotic contents of guard cells: stomates open or close when guard cells gain or lose turgor according to changes in the vacuolar concentration of K salts. Previous investigators have shown that Cl^- can serve as the counter ion for K^+ during stomatal movements but the extent of its participation has been observed to vary between 5% (13) to 100% (26). Malate has also been observed to accumulate in guard cells during stomatal opening. In order to study further the role of malate as an osmoticum an enzymatic assay in which malate is oxidized and NAD is reduced was modified from a procedure described by Goldberg and Passonneau (10). Since the NADH thus formed is measured fluorimetrically, small quantities of malate (10^{-6} M) can be detected.

The assay has been used to study malate accumulation during stomatal openings and how this is affected by the presence of external Cl^-. Stomates on epidermal strips supplied with K^+ and immodiacetate, an impermeant Zwitterion, form malate as they open; the increases in malate follow a nearly linear relationship with aperture. However, when epidermal strips are supplied with KCl, this relationship between

malate and stomatal aperture breaks down. External Cl^- also reduces stomatal sensitivity to external CO_2. These effects of Cl^- on malate formation and on CO_2-sensitivity are interpreted as resulting from different methods for regulation of cytoplasmic pH.

The assay was also used to investigate the fate of malate during ABA-induced closure. It was found that a large proportion (up to 80%) of the malate cumulated during opening was released to the bathing solution following the application of 10^{-5} to 10^{-7} M(±)ABA to epidermal strips.

MALATE CONTENT OF LEAF EPIDERMIS IN RELATION TO STOMATAL APERTURE AS EFFECTED BY EXOGENOUS CHLORIDE

By

Carol Anne Van Kirk

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Botany and Plant Pathology

1977

ACKNOWLEDGMENTS

To my major professor, Klaus Raschke, and to the other members of my guidance committee, Drs. Deborah Delmer, Norman Good, Hans Kende, and Anton Lang, for helpful discussion and useful criticism of the manuscript.

To Drs. Philip Filner and Clarence Suelter, for the use of their spectrophotoflurometers;

To Don Richman, for assistance in touching-up the figures;

And to Dr. Lionel Rosen, for helping me to see, and thus, help myself.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

PIPES	Piperazone-N-N'-bis(2-ethane sulfonic acid)
MES	2(N-morpholino)ethane sulfonic acid
IDA	Iminodiacetic acid
PVP-40	Polyvinyl pyrrolidone
APAD	Acetyl pyrridone adenine dinucleotide
EDTA	Ethylenedramine tetraacetate
NAD	Nicotinamide adenine dinucleotide
MDH	Malate dehydrogenase
PEP	Phosphoenolpyruvate
OAA	Oxaloacetate

INTRODUCTION

Stomatal aperture is controlled through regulation of guard cell turgor. Guard cell turgor is, in turn, determined by the vacuolar contents of K salts or Na salts (22), in the case of some halophytes. The K salts used by guard cells can be either organic, primarily K malate, or inorganic, primarily KCl. Organic acid metabolism has been implicated in controlling stomatal turgor in addition to its role in producing organic osmotica (24). Labelling studies with ^{14}carbon have provided much information on organic acid metabolism in guard cells (6,7,25). However, in these experiments it was estimated that less than 0.05% of the total malate pool became labelled. Hence, in order to study overall general changes in malate, labelling studies must be supplemented by other methods for detecting malate. To this end, an enzymatic assay for determining small quantities of malate in epidermal tissue was modified from a procedure developed by Goldberg and Passonneau (10). The assay has been used to investigate how availability of Cl^- alters malate accumulation as well as determining, in part, the fate of malate upon stomatal closure.

MATERIALS AND METHODS

Experimental Material

Plant Material

Vicia faba improved Long Pod variety from Lagomarsino Seed Co., *Commelina communis* (T. A. Mansfield University of Lancaster, Britian), and *Commelina coelestis* (Botanical Gardens, Tübingen, Germany) were grown either in a glasshouse (temperature: 23-29°C; humidity: 70-80%; light supplemented with Sylvania Gro-Lux fluorescent tubes to give 30 μW cm^{-2} to extend the natural light period to 20 hours) or in a growth chamber under 8.5 mW cm^{-2} for 16 hours, 85% relative humidity, and a temperature of 27°C during the light period and 23°C during the dark period.[1] The soil consisted of 2 parts baccto potting soil (Michigan Peat, Houston, Texas) and 1 part perlite (W. R. Grace & Co.). The plants were grown in 10 cm square plastic pots with a soil depth of about 12 cm. Once a week, the plants were watered with freshly prepared Hoagland's solution, pH 6.5; on the other days deionized water was used. In early experiments, plants were watered on alternate days with ½ strength Hoagland's solution or deionized water, and *C. communis* was grown in 36 x 27 cm plastic flats in a gravel-soil mixture at a depth of about 3 inches. *Tulipa gesneriana* leaves were obtained on the day

[1] No differences in experimental results could be correlated with different growth conditions.

of an experiment from Dr. A. De Hertogh of the Horticulture Department at Michigan State University.

Preparation of Leaf Sections

For experiments on V. faba, C. communis, or C. coelestis, 2nd, 3rd, and/or 4th fully expanded leaves from 3-5 week-old plants which had not yet flowered were cut at the petiole with a razor blade in the morning and placed in distilled, deionized water; for experiments on tulip, the lowest leaf was cut at the base and placed in distilled, deionized water. Within 15 min, the leaves were rinsed, the upper and lower surfaces were wiped with moist tissue paper, and the leaves were cut into sections of 1-3 x 2-4 cm^2. The sections were rinsed at least 3 times with water and floated abaxial side up on distilled, deionized water.

Preparation of Epidermal Strips

Epidermal strips were made by peeling an area of approximately 5 x 10-15 mm^2 from the lower epidermis of leaf section with dissecting forceps. V. faba strips were peeled at an obtuse angle which caused 60-80% of the epidermal cells to rupture; in other species, the epidermis is tougher and the epidermal cells remain intact. The strips were rinsed 5-10 times in distilled water and any adhering mesophyll cells were removed by rubbing a dissecting needle along the underside of the strip.

Experimental Procedures

Flow-through Chamber

Early studies on the interaction of Cl^- and CO_2 were conducted on epidermal strips of C. communis. On the morning of the day of an experiment, the 2nd, 3rd, and/or 4th fully expanded leaf was removed and leaf sections were made as described above. The sections were floated on distilled, deionized water, abaxial side up, and placed under 8.5 mW cm^{-2} light and CO_2-free air to open the stomates. After approximately 4 hr, epidermal strips were made. The strips were rinsed 5-10 times in distilled, deionized water and mounted cuticular side up on a Plexiglass slide having a central well 2 mm in diameter. A small glass slide covered the strip and O_2-saturated 10 mM PIPES buffer, pH 6.8, containing different amounts of KCl, was passed over the strip at a rate of 0.55 ml min^{-1}. The chamber was mounted on the stage of a Zeiss microscope connected to a Sony video camera and a TV monitor. Stomatal apertures were measured on the TV screen using a ruler. If CO_2 was to be added, after 10-20 min, the required amount was mixed into the O_2 bubbling through the buffer by means of a cascading system of Wösthoff pumps (H. Wösthoff o, H. D. Bochum, Germany).

Experiments with Stomates Opened In Situ

In some experiments, experimental material consisted of epidermal strips whose stomates had been opened while the epidermis remained in contact with the mesophyll. Leaf sections were made as described and floated on H_2O, abaxial side up, under mercury vapor lamps suspended above a 5 cm H_2O filter (intensity: 8.5 mW cm^{-2}) and in a stream of

humidified air (flow rate: ~50 l h^{-1}) containing either zero or ambient (approximately 300 pμl l^{-1}) CO_2. Dark conditions were obtained by either covering the petri dish with a light-tight box or placing the dish in a drawer (in the latter case, no air was passed over the sections). After about 4-5 hr, epidermal strips were made and were mounted in water on microscope slides. The area of the strip was measured with a ruler and stomatal apertures were measured under the microscope using an eyepiece micrometer. The tissue was then plunged into boiling 80% ethanol, extracted, and assayed for malate as described below.

Experiments on Isolated Epidermis

In other experiments, stomates were allowed opened after isolation of the epidermis rather than in situ. The leaves were removed in the morning and cut into sections. The sections were floated on double distilled water in a large petri dish that was covered with aluminum foil to keep the sections under dark conditions and thus inhibit stomatal opening. Epidermal strips were made as described above. In this case, the strips were floated on double distilled water in the dark until all strips for one day's experiment were made (usually 40-60 min were required). The strips were then transferred to 2 ml of treatment solution that was contained in a disposible 5 ml plastic beaker. The solutions contained 10 mM MES buffer, pH about 5.6, 90-110 meq l^{-1} K^+, and Cl^- at concentrations varying from zero to 100 meq l^{-1}; iminodiacetate (IDA), a presumably impermeant Zwitterion, balanced K^+ not balanced by Cl^-.[1] The relative proportions of K^+, Cl^-, and IDA were

[1]This compound has been used in earlier experiments in this capacity (22).

attained as follows: For solutions containing between zero and 10 meq Cl^- l^{-1}, 100 mM KIDA was prepared by titrating 0.1 N KOH and 10 mM MES with IDA, allowing sufficient time for the IDA to dissolve. Then KCl was added to supply the Cl^- in varying amounts as required. When 100 meq Cl^- l^{-1} were required, 100 mM KCl was added to a solution of 10 mM MES; the pH was adjusted with KOH. The beakers were placed in plastic petri dishes which were then clamped shut and placed in the same light conditions used for experiments on leaf sections. The petri dishes had one air inlet, and several air outlets; humidified air containing either ambient or zero CO_2, was forced through these dishes at a flow rate of 12 l hr^{-1}. The petri dishes were kept under these conditions for 4 hr. Areas and apertures were then measured and the tissue was transferred to boiling ethanol for extraction.

Extraction

Malate was extracted from epidermal tissue in 3 changes of boiling 80% alkaline ethanol (alkalinity achieved by adding 5 drops of 2N NaOH l^{-1} 80% ethanol).[1] The volume of each change was about 5 ml and each was boiled for 5-10 min. The tissue was killed by boiling in the first change of ethanol for 1-2 min; extraction was completed after a number of samples had been collected. The extracts were evaporated to dryness in a water bath at 50°C. Filtered air was bubbled through the extracts to speed evaporation. The evaporated extracts were kept in a

[1]It was observed that 80% ethanol containing a few drops of either HCl or NaOH resulted in higher malate, about 8% and 12% respectively, per unit area of epidermal tissue than pure 80% ethanol.

freezer until they were resuspended in either 2x distilled water or 0.2 M hydrazine buffer, pH 9.0, and assayed for malate. This procedure was checked by "extracting" a known amount of malate, evaporating to dryness, resuspending, and assaying; recoveries varied between 96 and 102%.

Malate Assay

Malate was assayed by enzymatic analysis according to a procedure adapted from Goldberg and Passonneau (10). In this method, malate is oxidized to oxaloacetate (OAA) with concomitant reduction of NAD by the action of malate dehydrogenase (MDH). The equilibrium of this reaction lies very much in favor of OAA reduction rather than malate oxidation. To overcome this problem, the reaction is run in the presence of hydrazine which traps the OAA formed as the hydrazone derivative and thus prevents the back reaction. In addition, running the reaction at pH 9.0 shifts the equilibrium in favor of malate oxidation (10,38). The NADH formed was measured fluorimetrically using an Aminco Bowman Spectrophotofluorometer (Model #XLS 1AM2). The exciting wavelength was 340 nm and emission at 470 nm was monitored. All entrance and emission slits were set at 1 mm; the photomultiplier tube entrance slit was 2.5 mm. The instrument was set for maximum sensitivity.

The reaction was run in a total volume of 2.005 ml. The medium consisted of 0.2 M hydrazine buffer, pH 9.0 (hydrazine hydrate was obtained from J. T. Baker Chemical Co. and from Mallinckrodt), 0.12 mM NAD (Sigma, Grade V),[1] 0.2 mM EDTA (Fisher Chemical Co.). Samples of

[1]Initially, Grade III NAD was used but it was found that Grade V resulted in a lower background fluorescence.

evaporated epidermal extract resuspended in 0.2 M hydrazine buffer, pH 9.0, were included; the volume of the aliquot was varied according to the estimated concentration of the sample but was usually 0.2 ml. To start the reaction, 5 µl of MDH (Sigma, Grade III), which provided 0.05 units of enzyme per assay, were added. Fluorescence was read before that start of the reaction and after 1 hr incubation at room temperature following the addition of the enzyme. Each sample was assayed in duplicate as was sample plus a known quantity of malate (Eastman Organic Chemical Co.), which was used for calibration. The percent conversion of malate in the assay was measured against a known amount of NADH (Sigma, Grade III). Nanogram quantities of malate could be measured with this method.

Goldberg and Passonneau suggest that acetyl pyridine adenine dinucleotide (APAD) be used as a cofactor for MDH rather than NAD, presumably because of its higher oxidation potential (38). However, excitation and emission spectra for reduced APAD showed that wavelengths for maximum excitation and emission were 370 nm and 492 nm, respectively, and not 340 nm and 470 nm, as implied in the assay description. Since the absorbancy of the resuspended plant extracts increases greatly at wavelengths longer than 350 nm, it was decided to use NAD instead of APAD.

In other modifications of the method of Goldberg and Passonneau, it was found that doubling the hydrazine concentration from 0.1 to 0.2 M increased the conversion of malate. In addition, tris-albumin, which Goldberg and Passonneau include to "stabilize" MDH, was found to be

without effect with my reaction mixture while it increased turbidity; therefore, it was decided to delete it from the assay. Finally, the volume of the assay was doubled from 1 to 2 ml to reduce the possibility of scatter-produced artifacts in measuring NADH fluorescence.

As modified, the assay gave about 60% conversion of malate. This was determined by comparing the fluorescence resulting from reacting a known quantity of malate in the assay with the fluorescence from a known amount of NADH. Coupling the MDH reaction with the transamination reaction catalyzed by glutamine oxaloacetate transaminase (in the presence of glutamine and the absence of hydrazine to produce aspartate rather than OAA-hydrazone) did not enhance malate conversion.

A number of techniques were tested for their effectiveness in removing epidermal "contaminants", most probably phenolics, that were extracted along with malate. These compounds posed problems for quantifying malate (31) because they fluoresced, as indicated by background fluorescence in the assay reaction mixture, in addition to absorbing both the exciting and emitting wavelengths of NADH, as shown by the absorption spectra of resuspended extracts. Extraction in boiling water rather than 80% ethanol did not improve this situation. Partitioning against methylene chloride was useless because the phenolic compounds moved with malate. Filtering through charcoal or amberlite columns was inadequate because malate itself bound tightly to charcoal while, with amberlite, both the phenolics and malate came through with the void volume. Purification with ion exchange columns according to

the method of Canvin and Beevers (2) was found to be unsatisfactory because the Dowex contained fluorescing compounds which extensive prewashing did not remove; also, the method was time-consuming. Polyvinylpyrrolidine did seem to absorb some of the phenolic compounds. However, residual PVP remaining in the extract after centrifugation increased the turbidity of the assay reaction medium. It was finally decided that, with appropriate calibration, the resuspended extracts could be assayed directly.

Because the relative concentration of "contaminants" varied from extract to extract, it was necessary to calibrate each sample for malate. This was done by adding a known quantity of L-malate to the assay mixture. The NAD conversion in this reaction mixture was then compared with that observed in an identical reaction mixture but without the malate "spike". The amount of malate originally present in the aliquot of extract was then determined according to the following equation:

$$c = \frac{F_2 - F_1}{F_3 - F_2} \times C'$$

where F_1 = Fluorescence of assay mixture before addition of enzyme

F_2 = Fluorescence of assay mixture containing resuspended extract 1 hr after adding MDH

F_3 = Fluorescence of an assay mixture containing both resuspended extract and a malate "spike" 1 hr after adding MDH

C' = concentration in mol l^{-1} of the "spike"

and c = concentration of mol l^{-1} of the resuspended extract.

This equation holds as long as the fluorescence is linearly related to concentration.

RESULTS

Malate in Epidermis of Leaf Sections

Preliminary experiments in which leaf sections were floated on distilled water in the light or in darkness, and in the presence or absence of CO_2 (different treatments to vary stomatal aperture), showed that generally, as stomates opened, the level of malate in the lower epidermis of C. communis, V. faba, and T. gesneriana increased. However, large variations in both the amount of malate initially present in the tissue, and in the epidermal contents after exposure to the different treatments, were observed. The scatter was seen both in experiments conducted on a given day and between experiments of different days. For example, out of 10 experiments on C. communis, the malate content of the epidermis increased 4-10 fold during stomatal opening (from, on the average, 20-50 pmol mm^{-2} to 120-400 pmol mm^{-2}) in 6 experiments but no increase, or only a slight one, was seen in the other 4 experiments. In 2 of these, virtually no malate was detected regardless of stomatal aperture while in the other 2, epidermal malate was relatively high whether or not stomates were open. Even in some of the experiments where epidermal malate was seen to increase, a few samples of epidermis with closed stomates contained almost as much malate as samples with open stomates. In 5 experiments on tulip, the results were generally

more consistent, although in this species, too, variable malate levels were detected. Usually, epidermis with closed stomates contained little (20-50 pmol mm^{-2}) or no detectable malate while, when stomates were open, about 300-400 pmol malate mm^{-2} was commonly observed. In one experiment using C. coelestis, epidermis, with stomates at 14 μm aperture, contained about 475 pmol malate mm^{-2} but in epidermis with stomates at 0 μm, about 375 pmol malate mm^{-2} was measured.

Two possible explanations for this variation are:

(1) When these measurements were made, the assay and extraction procedures were being developed; thus, the scatter could have been caused by faulty technique.

(2) The utilization of malate as a counter ion for K^+ could be affected by endogenous factors within the leaf, such as the availability of inorganic anions. This could result in both inter- and intra-species variation but it would not explain the high malate content sometimes seen when stomates were closed.

KCl and Sensitivity to CO_2 in Epidermis of C. communis

As part of some introductory work on stomates, and in an attempt to verify the results obtained from Xanthium strumarium (23), I chose to investigate CO_2 sensitivity in C. communis. In this study, stomatal aperture was monitored while 10 mM PIPES buffer, pH 6.8, containing either dissolved O_2 or dissolved O_2 plus CO_2, was passed over isolated epidermes. Because it had been reported that the presence of KCl

resulted in more uniform apertures (32), small amounts of KCl were added to some of the buffer solutions.

The results of experiments conducted on 2 different days are shown in Figures 1 and 2. It can be seen that the presence of KCl changed stomatal responses to CO_2. In Figure 1, when 400 l l^{-1} CO_2 was bubbled through the buffer in the absence of CO_2, stomatal aperture declined by about 30% on the average. With 10^{-4} M KCl in the buffer, one stoma decreased its pore size by about 20% and 3 others remained essentially unchanged while in 10^{-3} M KCl, stomatal apertures were either nearly constant or increased slightly. Controls exposed to neither CO_2 nor to KCl (data not shown) generally exhibited slight increases in aperture during similar time periods. When the quantity of CO_2 bubbling through the buffer was increased to 1,000 µl l^{-1}, a similar pattern was observed (Figure 2). However, the concentration of KCl necessary to suppress CO_2-induced closure increased: when no KCl was available, the average closing rate was 0.011 relative aperture units min^{-1};[1] with 10^{-3} M KCl, the rate dropped slightly to about 0.010 relative units min^{-1}; 10^{-2} M KCl resulted in a large slow-down in the closing rate to about 0.002 relative units min^{-1}. Clearly, the presence of KCl diminished CO_2 sensitivity in epidermal strips of C. communis. At the time of these experiments it was not known whether or not this was due to the salt, to K^+ or to Cl^-. Later experiments indicate that the decline in CO_2 sensitivity when KCl was available results from effects of Cl^-.

---

[1] Relative aperture defined as $\frac{\text{aperture}}{\text{maximum aperture}}$.

Figure 1. The effect of KCl on stomatal response to 400 µl l^{-1} CO_2 in epidermal strips of C. communis.

Epidermal strips were mounted in a Plexiglass chamber under a microscope, connected with a Video camera and TV monitor, and 10 mM PIPES buffer, pH 6.8 containing different concentrations of KCl, and through which 100% O_2 was bubbled, was passed through the chamber. Stomatal apertures were measured on the TV screen. At the time indicated by the arrow, 400 µl l^{-1} CO_2 was added to the O_2. A. 10^{-3} M; B. 10^{-4} M KCl; C. no KCl. When KCl is present stomates lose their sensitivity to CO_2.

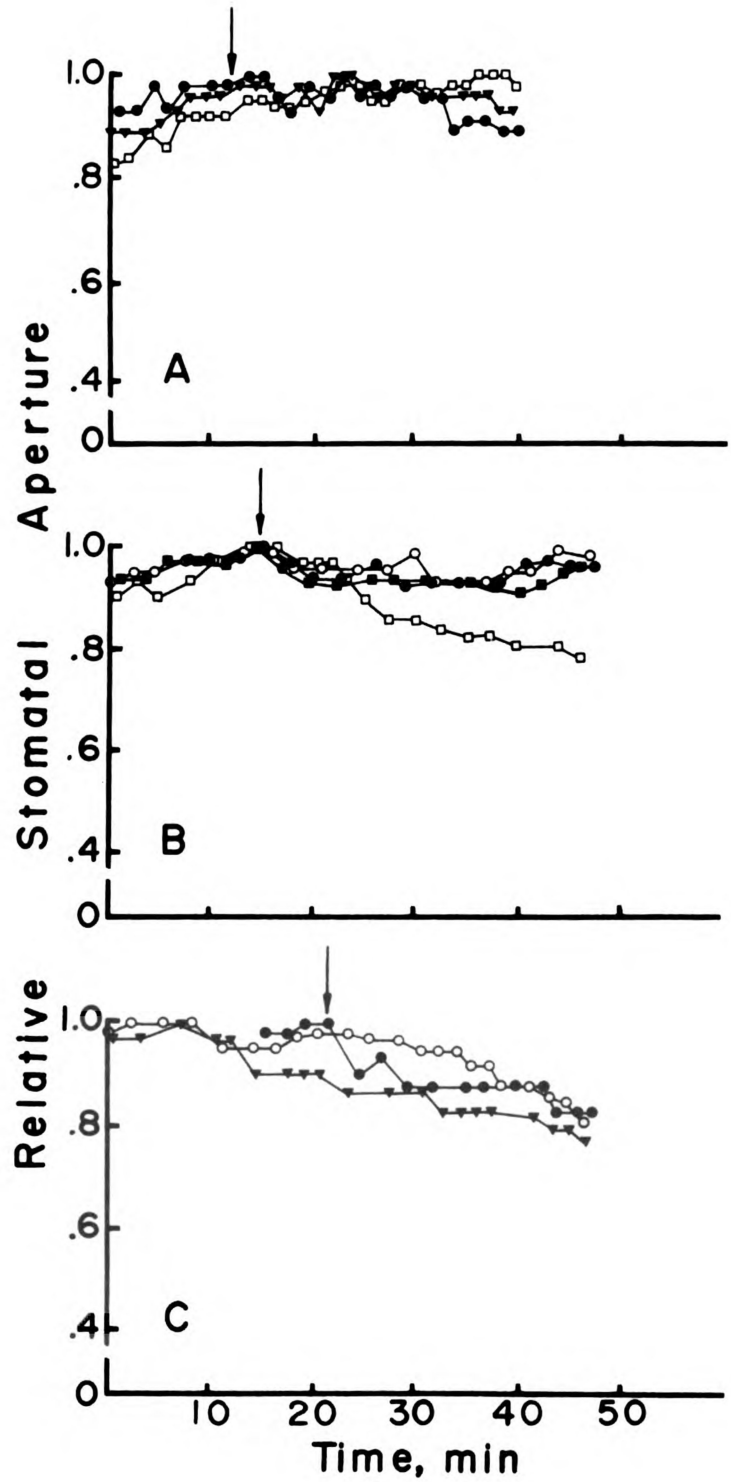

Relative Stomatal Aperture
1.0
.8
.6
.4
0
A
B
C
10
20
30
40
50
Time, min

Figure 1

Figure 2. The effect of KCl on stomatal response to 1,000 µl l^{-1} CO_2 in epidermal strips of C. communis.

Procedure followed as described under Figure 1 except at the time indicated by the arrow, 1,000 µl l^{-1} CO_2 was added to the O_2 bubbling through the buffer. A. 10^{-2} M KCl, B. 10^{-3} M KCl; C. No KCl. When KCl is present stomates lose their sensitivity to CO_2.

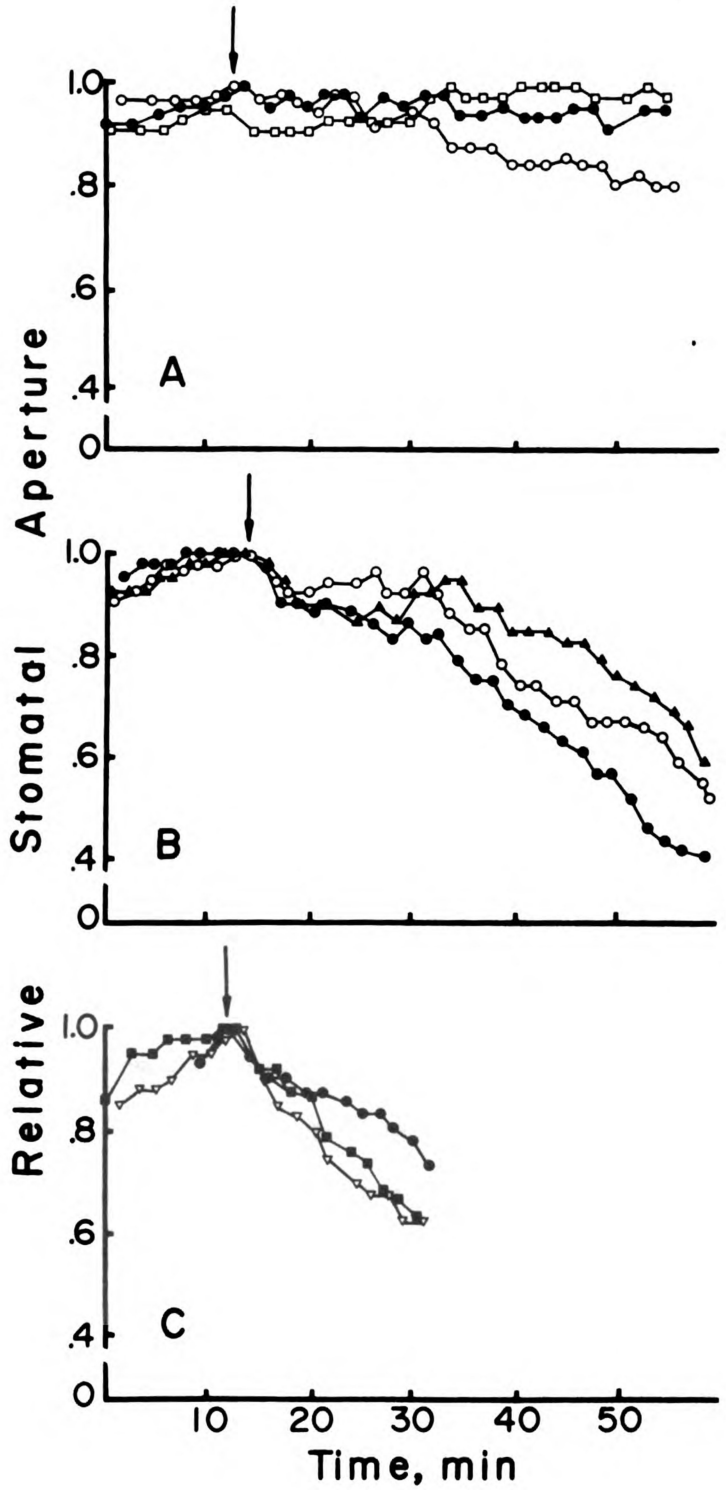

Relative Stomatal Aperture
1.0
.8
.6
.4
0
A
B
C
10
20
30
40
50
Time, min

Figure 2

Malate in Epidermal Strips: Effects of Cl^- and Its Interaction with CO_2

In order to investigate possible malate/Cl^-/CO_2 interactions, epidermal strips of V. faba were floated on solutions of about 100 meq l^{-1} K^+, 10 mM MES, pH about 5.6, and varying amounts of Cl^-. The K^+ concentration was kept high and nearly constant in order to eliminate effects on stomatal aperture resulting from K^+ availability. IDA, an impermeant Zwitterion, was added to balance K^+ not balanced by Cl^-. The strips were then exposed to light and to air containing either zero or ambient (normally taken to be about 300 µl l^{-1}) CO_2 for 4 hr. A summary of the results of 14 separate experiments is shown in Figure 3. Figure 3A shows that as stomates attained their various apertures in the absence of Cl^-, the malate level increased in a nearly linear fashion. But, as can be seen in Figure 3B, when Cl^- is present at 10^{-5} to 10^{-1} eq l^{-1}, this relationship between malate and aperture breaks down. Thus, the ready availability of an absorbable inorganic anion seems to make the formation of organic counter-ions for K^+ unnecessary. The variation in stomatal aperture, which is seen in both parts of Figure 3, results from 1) endogenous variation in the tissue and 2) the presence or absence of CO_2. Most of the data for small apertures in Figure 3A were obtained with strips that were analyzed directly after the leaf sections were peeled.

At no point were the stomates completely closed; neither did the epidermal malate content fall to zero. If one assumes that all malate is concentrated in the guard cells (which seems reasonable since 80-100% of the epidermal cells appeared to have ruptured by the conclusion of

Figure 3. Malate content of isolated epidermis of V. faba related to stomatal aperture.

Epidermal strips were removed from leaf sections in the morning when the stomates were almost closed. The strips were floated on buffer in the light for 4 hrs, and air containing either zero CO_2 or ambient (approximately 300 $\mu l\ l^{-1}$). CO_2 was passed over the strips. After 4 hrs, apertures were measured under the microscope and the tissue was extracted and assayed for malate as described in Materials and Methods.

A. Strips exposed to 10 mM MES buffer, pH about 5.6, and 100 mM KIDA in the absence of Cl^-; epidermal malate increases linearly with increases in stomatal aperture. B. Strips exposed to 10 mM MES buffer, pH about 5.6, 90-110 meq $K^+\ l^{-1}$, and Cl^- at 10^{-5} to 10^{-1} eq l^{-1} with IDA added to balance K^+ not balanced by Cl^-; in the presence of Cl^-, the linear relation seen in A breaks down.

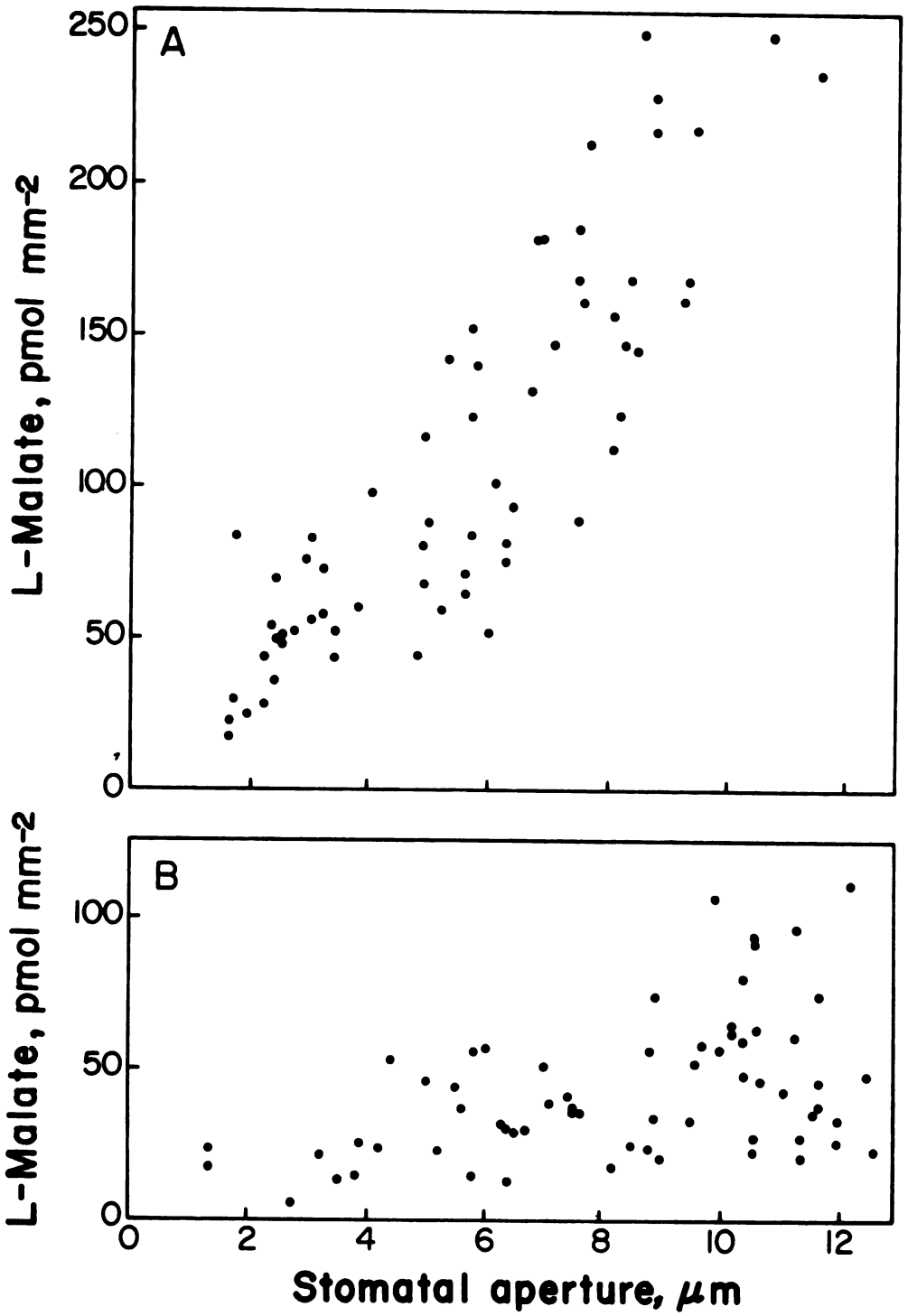

A
B
L-Malate, pmol mm-2
L-Malate, pmol mm-2
250
200
150
100
50
0
100
50
0
0
2
4
6
8
10
12
Stomatal aperture, μm

Figure 3

the 4 hr period), one can estimate that, in the absence of Cl^-, malate accumulation was sufficient to balance approximately 2/3 of the K^+ previously measured in guard cells of V. faba comprising open stomates (13); presumably, the K^+ not balanced by malate was present as KCl.[1]

As shown in Figure 4, no correlation between the amount of Cl^- offered and the quantity of malate formed within the epidermis was observed. However, even with Cl^- concentrations as low as 10^{-5} eq l^{-1}, malate accumulation was suppressed. With Cl^- at 10^{-2} or 10^{-1} eq l^{-1}, there is somewhat less scatter in the data of Figure 4, but there were also fewer treatments at these higher concentrations.

In comparing Cl^- offered to the epidermal strips and the final aperture attained by their stomates (Figure 5), much scatter is also evident. If the mean of the final apertures measured is plotted as a function of the logarithm Cl^- concentration, then, between 10^{-5} and 10^{-3} eq Cl^- l^{-1}, a nearly linear increase in aperture with increasing Cl^- concentrations is seen; this increase appears to saturate at 10^{-3} eq Cl^- l^{-1}. However, the extreme scatter in these data makes this conclusion tenuous at best.

Table I summarizes the results of these same experiments with respect to CO_2 sensitivity. Again, there is scatter in the data but, as previously observed with C. communis, exogenous Cl^- reduces stomatal responsiveness to CO_2. In these experiments, 10^{-2} eq Cl^- l^{-1} were

---

[1] I tried to follow changes in the Cl^- content of guard cells using the Cl^- stain adapted by Raschke and Fellows (26). However, at best, this stain is only qualitative, and definitive results could not be obtained.

Figure 4. Malate accumulation in epidermal strips of V. faba in relation to the concentration of external Cl^-.

Same data as in Figure 3B, page 20. o strips exposed to CO_2-free air; • strips exposed to CO_2-containing air. There is no obvious relation between the concentration of external Cl^- and the quantity of malate found in the epidermis.

Figure 5. Final aperture in epidermal strips of V. faba in relation to external Cl^- concentration.

Same data as in Figure 3B, page 20. o strips exposed to CO_2-free air; • strips exposed to CO_2-containing air. There is no obvious relation between final stomatal aperture attained and the concentration of external chloride.

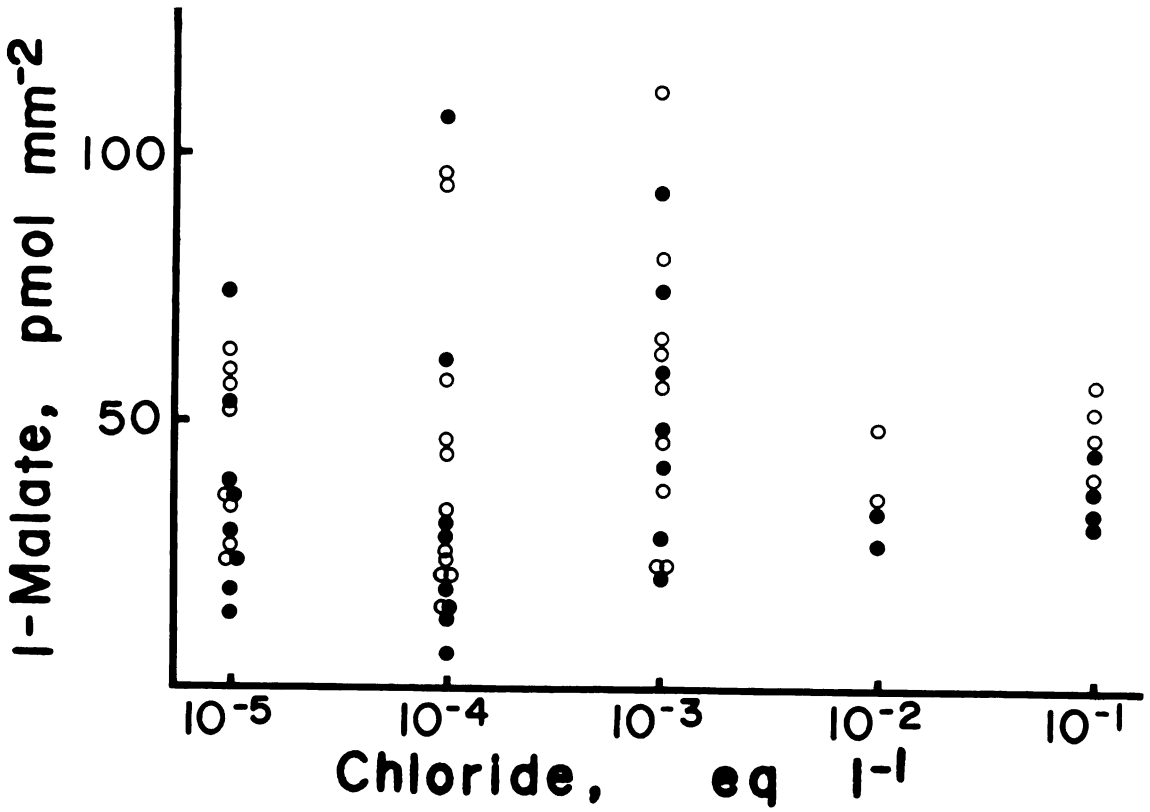


Figure 4

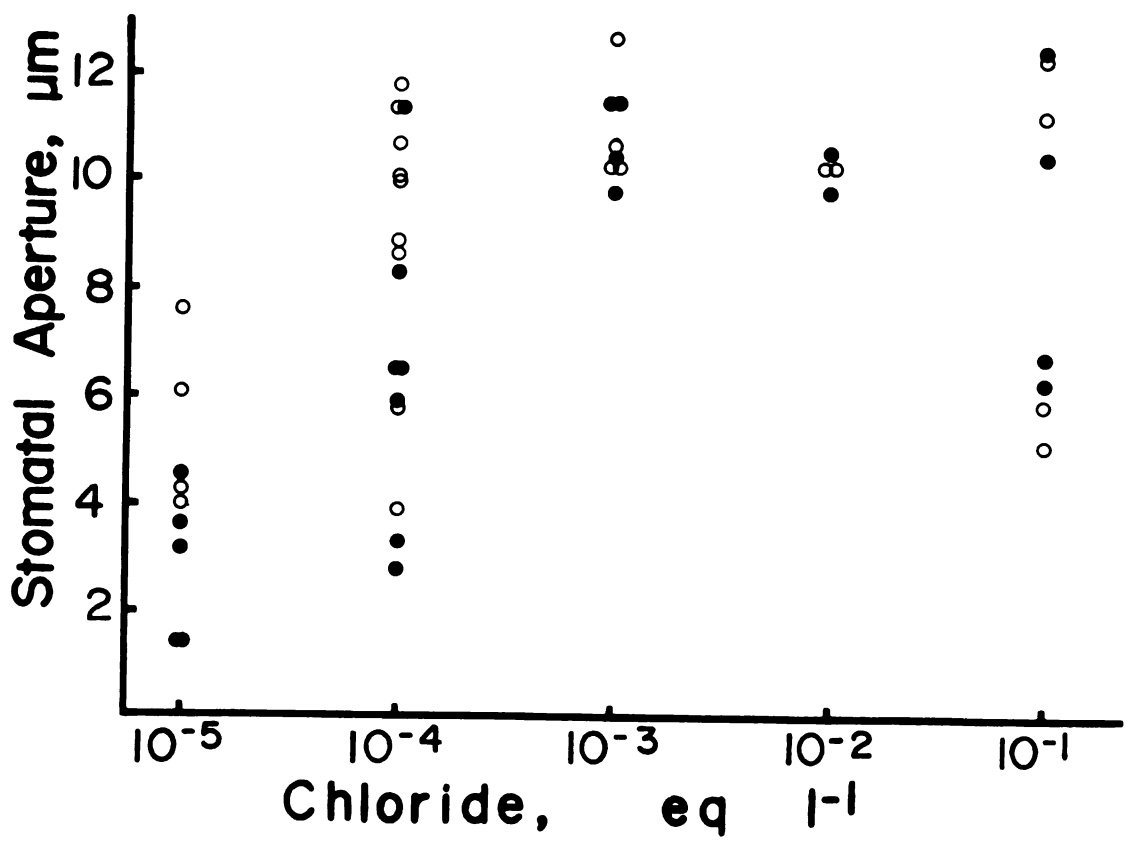


Figure 5

Table I. The effect of external Cl^- on sensitivity of stomates to CO_2 in isolated epidermis of V. faba. Strips exposed to air containing either zero or ambient CO_2 in light, 90-110 meq $K^+ l^{-1}$, 10 mM MES buffer, pH about 5.6, and various concentrations of Cl^- for 4 hr. Data taken from Figure 3, page 20. Decrease in aperture = aperture in the absence of CO_2 - aperture in the presence of CO_2. Cl^- reduces stomatal sensitivity to CO_2.

Cl^-, eq l^{-1}	Decrease in Aperture
0	2.43 ± 1.44
10^{-5}	1.20 ± 2.33
10^{-4}	1.48 ± 1.65
10^{-3}	1.25 ± 1.23
10^{-2}	0.00 ± 0.83
10^{-1}	-0.35 ± 0.75

required to abolish CO_2 sensitivity; with 10^{-1} eq Cl^- l^{-1}, stomates were, on the average, more open in the presence of CO_2. However, in isolated instances, CO_2 insensitivity was observed even in the minus Cl^- controls. The scatter may, again, result from inherent variation in the tissue possibly resulting from different quantities of endogenous Cl^-.

The results of the preliminary experiments conducted on leaf sections had, in general, resulted in detection of higher levels of epidermal malate than were usually observed for epidermis whose stomates had been allowed to open following isolation from the leaf. I wished to see whether or not this was an artifact resulting from problems involved in working out the assay and extraction procedures. Therefore, in some experiments reported in this section, leaf sections of V. faba were floated on double distilled water in light and CO_2-free air for 4 hr. The lower epidermis was then isolated, adhering mesophyll cells were removed, stomatal aperture and tissue area were measured, and malate was extracted and assayed as described for experiments with epidermal strips. As shown by open circles in Figure 6 (compare with closed circles which represent the data of Figure 3A), epidermal malate was almost always greater in these tissues than in corresponding epidermis whose stomates had been allowed to open after stripping. The increase in malate was variable, from about 25% to 100%. Also, stomates which were allowed to open while the epidermis was in situ generally exhibited wider and more uniform apertures. This same experiment (i.e., comparing epidermal malate accumulation in leaf sections with that in

Figure 6. Epidermal malate in V. faba after stomates were allowed to open in leaf sections (o) or in isolated epidermis (●).

Data for isolated epidermis is the same as in Figure 3A, page 20. Leaf sections were floated on distilled water in the light for 4 hr; CO_2-free air was passed over the sections. Epidermal strips were then made. Stomatal aperture was measured under the microscope and the epidermis was extracted and assayed for malate as described in Materials and Methods. On leaf sections stomates attain a wider aperture and the concentration of malate in the epidermis continues to increase.

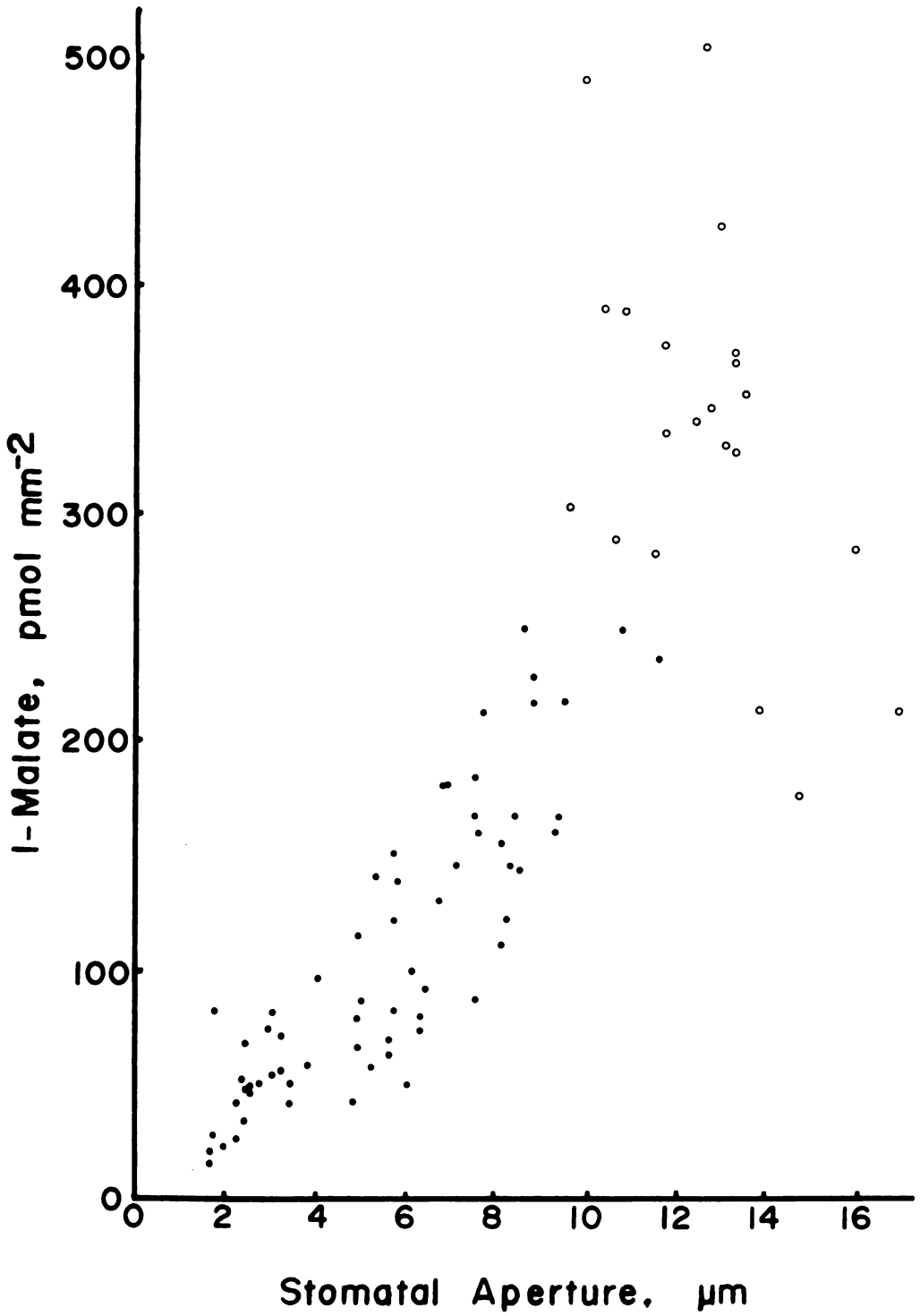

500
400
300
200
100
0
l-Malate, pmol mm-2
0
2
4
6
8
10
12
14
16
Stomatal Aperture, µm

Figure 6

isolated stripe) was also performed using C. communis, with similar results (data not shown).

To conclude this series of experiments, I wanted to determine whether or not the effects of Cl^- on malate accumulation could also be seen when leaf sections rather than epidermal strips were exposed to Cl^-. One experiment was performed. Leaf sections were floated on 100 mM KCl and stomatal apertures and the malate content of the epidermis after 5 hr in light and CO_2-free air were compared with apertures and epidermal malate in leaf sections floated on double distilled water. The results, shown in Figure 7, were consistent with observations made on epidermal strips: when Cl^- was available, less malate was accumulated. The decline in malate was approximately 50%. In leaf sections it is probable that not all stomates were equally exposed to the Cl^--containing solution which may, in part, explain why the malate content in these stomates was higher than that seen when isolated epidermis was exposed to Cl^-.

The Fate of Malate During ABA-induced Closure

Two types of experiments were performed to investigate the fate of malate upon stomatal closure induced by ABA. In the first, ABA, at 3 concentrations, was supplied to epidermal strips of C. communis taken from leaf sections with open stomates. After 30 or 40 min exposure to ABA in light and CO_2-free air, stomatal apertures were measured, and the tissue was extracted and assayed for malate. The solution on which the

Figure 7. The effect of 10 mM KCl on malate accumulation in the epidermis from leaf sections of V. faba.

Leaf sections were floated on distilled water in the light and in CO_2-free air (▲), on distilled water in the dark and in air containing ambient CO_2 (■), or on 100 mM KCl in the light and in CO_2-free air. After 4 hr, epidermal strips were made, stomatal apertures were measured, and the epidermis was extracted and assayed for malate as described in Materials and Methods. (o refer to measurements made on epidermis taken from leaf sections which were untreated.) As observed with isolated epidermis (Figure 3, page 20), Cl^- reduces the malate content of the epidermis.

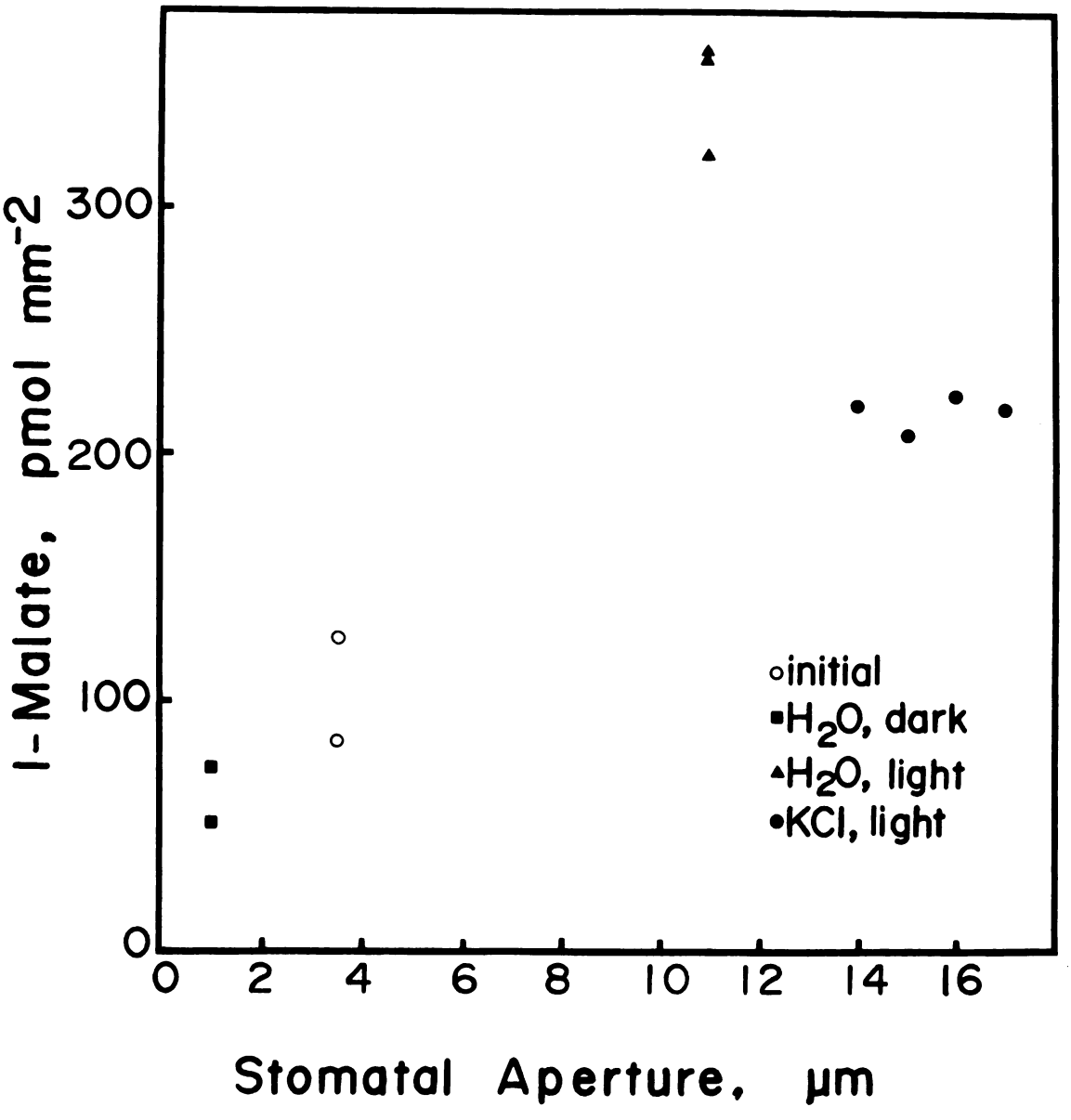

l-Malate, pmol mm-2
300
200
100
0
0
2
4
6
8
10
12
14
16
Stomatal Aperture, µm
initial
H2O, dark
H2O, light
KCl, light

Figure 7

strips had floated was also collected, evaporated, and analyzed for malate. The three concentrations of ABA supplied were chosen to produce different closing velocities.

Results of two separate trials of this experiment are shown in Table II. With increasing concentrations of ABA, stomates closed more rapidly, the malate remaining in the epidermis declined, and increasing amounts of malate were released to the bathing medium. In C. communis, epidermal cells remain intact after stripping; thus, malate released represents a net loss from the intact epidermis. The sum of malate remaining in the tissue and malate released to the solution increased with increasing degrees of closure. Strips floating on water also closed somewhat during the experiment. In this case, too, the malate content of the epidermis decreased from the initial level and malate appeared in the bathing solution.

In the second type of experiment, epidermal strips of V. faba, whose stomates were initially open, were floated on solutions of 10^{-4} M (+) ABA for periods of 15 min to 2 hr. After these times, apertures were measured and the tissue and bathing solution were analyzed for malate. The results, summarized in Figure 8, show that the malate content of the epidermis decreased essentially in parallel with stomatal aperture. It appeared that during the first 15 min, stomates did not exhibit much closure but the malate content decreased by about 25%; some of this (approximately 30%) appeared in the bathing solution. After the first 15 min, malate in the epidermis continued to decline while the concentration of malate in the bathing solution rose.

Table II. The Effect of ABA on Malate in C. communis Epidermis

Epidermal strips were made from leaf sections having open stomates and were floated on water or solutions of 10^{-5} to 10^{-7} M(±)ABA for the times indicated. Stomatal apertures were then measured and the tissue and the solutions were analyzed for ABA. As stomates close in response to ABA, the malate content of the epidermis declines and malate is released to the bathing solution.

	TREATMENT	APERTURE		MALATE, pmol mm^{-2}		
		Initial	Final	Tissue	Solution	Sum
Trial I: 40 min	Initial	16	-	551[1]		
	Initial	0	-	474		
	Water	15	0-11	165	165	330
	10^{-7} M(±)ABA	15	0- 5	146	203	349
	10^{-6} M(±)ABA	15	0	162	198	360
	10^{-5} M(±)ABA	15	0	77	357	434
Trial II: 30 min	Initial	14-16	-	381[1]		
	Initial	1- 5	-	243		
	Water	14-16	11-13	216	141	357
	10^{-7} M(±)ABA	14-16	0- 3	149		
	10^{-6} M(±)ABA	14-16	0- 2	117	165	282
	10^{-5} M(±)ABA	14-16	0	83	211	294

[1]For discussion of high levels of background malate, see pages 11 and 12.

Figure 8. The effect of 10^{-4} M(±)ABA on stomatal aperture, epidermal malate, and malate released to the bathing medium with time in V. faba epidermis.

Epidermal strips were made from leaf sections having open stomatis. They were floated in 10^{-4} M(±)ABA for the amounts of time indicated. Stomatal apertures were then measured and the epidermal tissue was extracted and assayed for malate as described in Materials and Methods. The ABA solutions in which the strips were floated were also assayed for malate.

▲ stomatal aperture; ● epidermal malate, o malate released. As stomates close in response to ABA, the amount of malate present in the epidermis decreases. Most of this malate appears in the bathing solution.

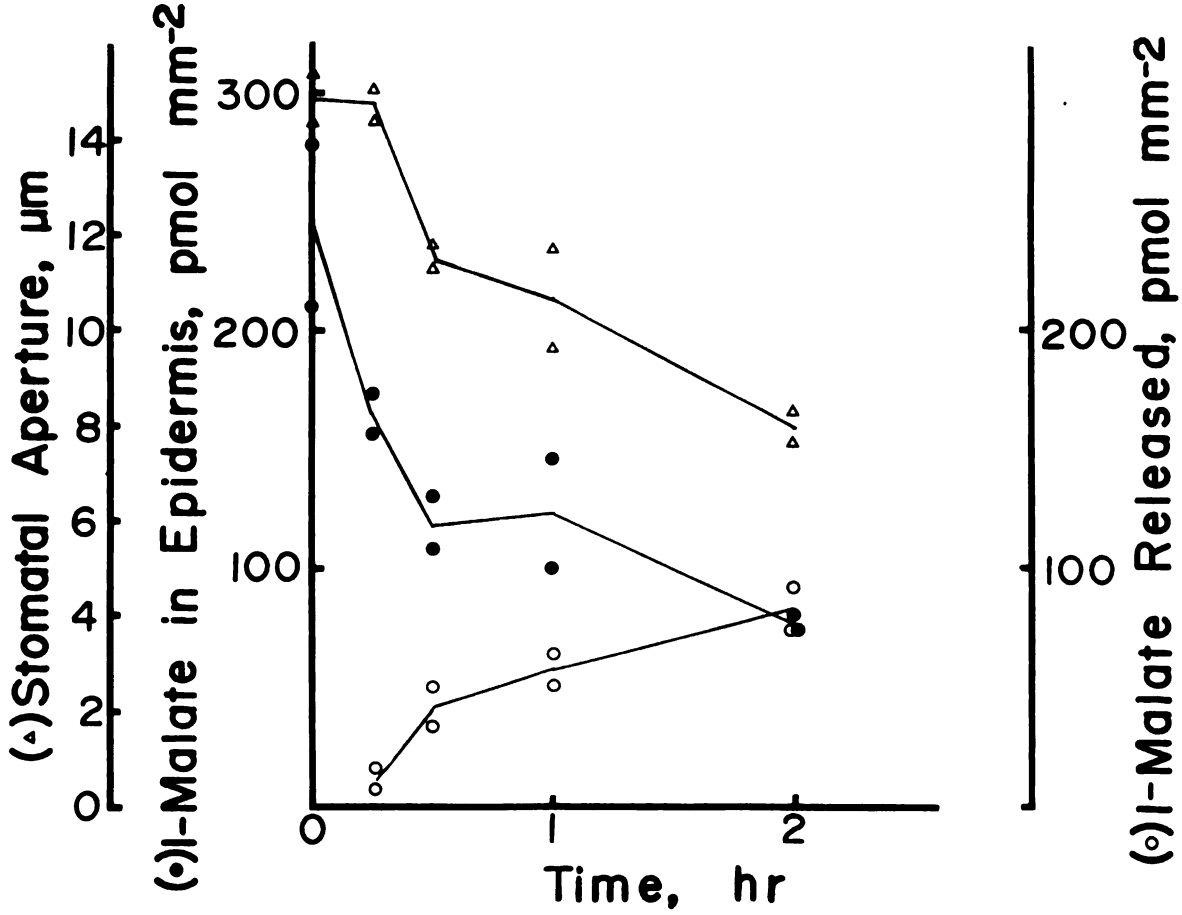

(▲)Stomatal Aperture, µm
0
2
4
6
8
10
12
14
(●)l-Malate in Epidermis, pmol mm-2
100
200
300
0
1
2
Time, hr
(○)l-Malate Released, pmol mm-2
100
200

Figure 8

Figure 8. The effect of 10^{-4} M(±)ABA on stomatal aperture, epidermal malate, and malate released to the bathing medium with time in V. faba epidermis.

Epidermal strips were made from leaf sections having open stomatis. They were floated in 10^{-4} M(±)ABA for the amounts of time indicated. Stomatal apertures were then measured and the epidermal tissue was extracted and assayed for malate as described in Materials and Methods. The ABA solutions in which the strips were floated were also assayed for malate.

▲ stomatal aperture; ● epidermal malate, o malate released. As stomates close in response to ABA, the amount of malate present in the epidermis decreases. Most of this malate appears in the bathing solution.

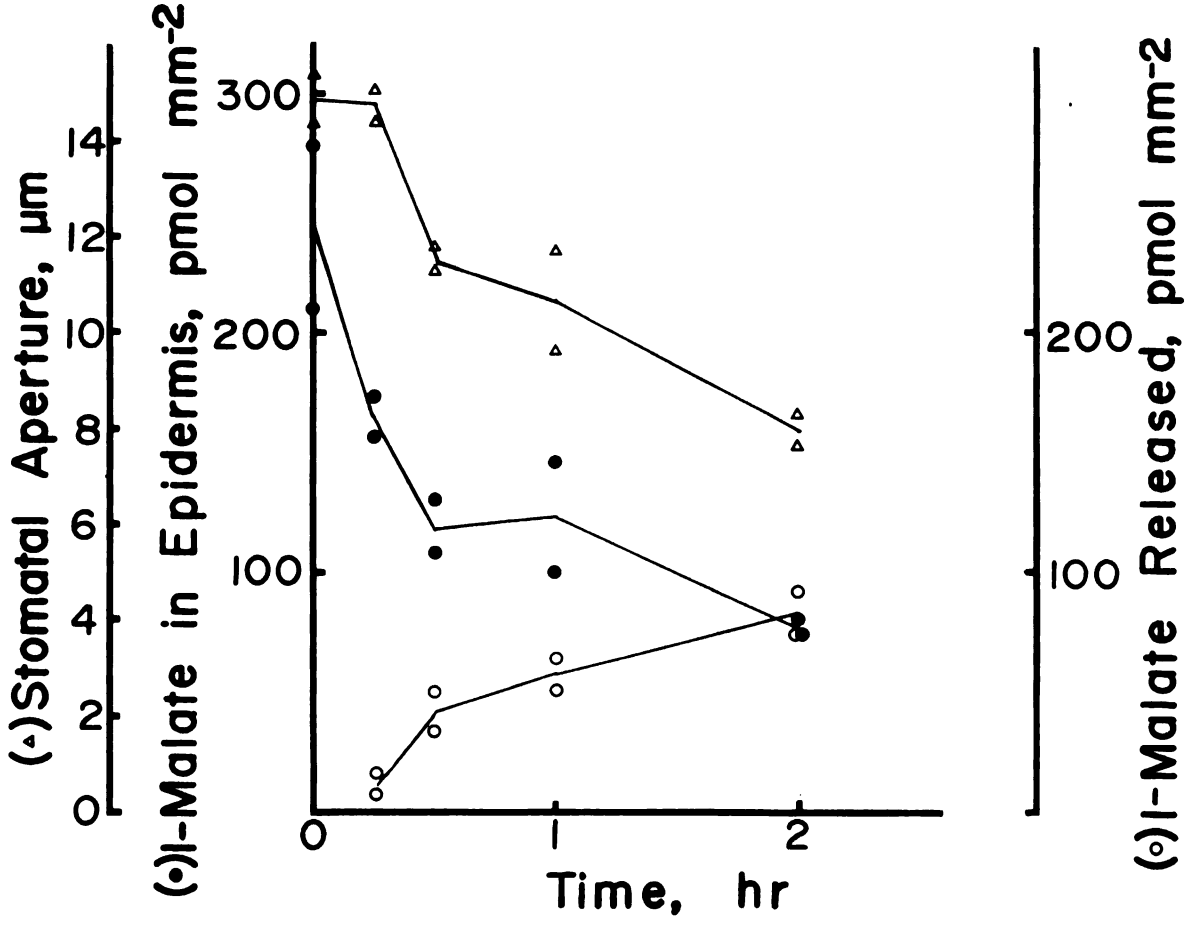

(▲)Stomatal Aperture, µm
0
2
4
6
8
10
12
14
(●)l-Malate in Epidermis, pmol mm-2
100
200
300
0
1
2
Time, hr
100
200
(○)l-Malate Released, pmol mm-2

Figure 8

Virtually all malate lost from the epidermis after the first 15 min could be accounted for by malate appearing in the solution. Thus, the guard cells seemed to rid themselves of osmotica primarily by expulsion.

DISCUSSION

Stomates open or close when guard cells gain or lose turgor because of changes in their osmotic contents (22,24). From the results obtained with tracer studies (8,9), and by direct measurement based on electron microprobe analysis (13,26,29) or K^+ electrodes (21), K salts have been shown to be taken up by guard cells in amounts sufficient to cause these turgor changes. (In some halophytes, Na^+ can substitute for K^+ (22).) However, there has been less certainty as to the anion which accompanies K into guard cell vacuoles. These techniques have shown that Cl^- can move with K^+ into guard cells during stomatal opening (4,13,18,21,26,28) but the extent of its participation has been observed to vary from about 5% in *V. faba* (13) to 100% in some individual stomates of *Zea mays* (26). In addition to this lack of evidence for the complete balance of K^+ by Cl^-—or some other inorganic anion—two observations suggested that organic anions might be involved:

1. When stomates open, the starch content is often seen to decrease (9).
2. When epidermal strips are floated on K salts of a non-absorbable acid (such as + iminodiacetatic acid), stomates open as widely as they open on KCl solutions (27).

The second observation suggests that the uptake of external anions is not necessary for stomatal opening and implies that guard cells can generate organic anions to balance K^+.

In 1973 Allaway reported that when stomates of V. faba opened, guard cells contained malate; the malate he detected could account for about half of the K^+ taken up during opening (1). Other work has supported Allaway's observations. Pallas and Wright (19) found that the organic acid content of V. faba epidermis increased upon stomatal opening. Pearson and Milthorpe (20) reported a nearly linear relationship between the level of epidermal malate and the stomatal aperture between 0 μm and 12 μm in C. cyanea. Quite recently Outlaw and Lowry (17), using enzyme amplification techniques, have demonstrated that similar increases in organic acids can be observed in isolated guard cells of V. faba.

Other work has implicated the guard cells themselves as the source of organic acids. Isolated epidermes of T. gesneriana and C. communis have been shown to incorporate $H^{14}CO_3$ (35) and $^{14}CO_2$ (6) into malate and aspartate. Epidermes of these species have relatively high activities of the enzymes necessary for carboxylation of phosphoenolpyruvate (PEP) to oxaloacetate (OAA), and its subsequent reduction to malate (37). In addition, autoradiograms of epidermes exposed to $^{14}CO_2$ show that the guard cells are the sites for CO_2 fixation (15,30,37).

My results are consistent with the previous work on malate accumulation during stomatal opening and the notion that this malate is formed in the guard cells. The quantities of malate found in the absence of Cl^- are in general agreement with the earlier reports. In preliminary experiments on 4 species, the malate content of the epidermis was found to increase in 12 out of 17 trials; in 5 of these

trials, virtually no malate was detected in the epidermis when the stomates were closed, while in the other cases the initial level was variable. The scatter, both in initial contents and in relative malate increases, could, in part, result from endogenous differences in the content of inorganic anions. Linearity in malate with aperture, as observed when stomates of V. faba strips were opened in the absence of Cl^-, would be expected due to unique properties of guard cell walls (22,24). In addition, the experiments in which stomates on isolated epidermes of V. faba opened while floating on K^+ solutions support the idea that the ability to synthesize malate resides within the guard cells themselves; the mesophyll cells were removed before the start of the opening treatment, and nearly all of the epidermal cells had ruptured by the end of the opening treatment (at least 60% were already ruptured at the start of the experiment). The earlier studies by other workers on malate accumulation were performed on epidermes whose stomates had been opened in situ.

Figure 6 compares the amount of malate I found when stomates opened on leaf sections with the amount I observed when stomates opened on strips floated on 100 mM KIDA. The scatter in these data preclude any statement concerning the role of the mesophyll in supplying stomates with malate or its precursors. If the mesophyll did supply the guard cells with malate or its precursors, one would expect to find wider stomatal apertures on leaf sections than on strips and a corresponding linear increase in malate. This situation is suggested by the data but the large variability in malate contents prevents drawing a definite conclusion.

Evidence from work by Dittrich and Raschke (7) supports the notion that the mesophyll does supply the epidermis with reduced carbon. They demonstrated that ^{14}C reduced by the mesophyll during photosynthesis can be detected in the epidermis. When ^{14}C-malate (6), ^{14}C-glucose-1-P, ^{14}C-glucose, ^{14}C-sucrose, or ^{14}C-maltose were offered to isolated epidermes, malate became labelled. Incorporation of $^{14}CO_2$ by epidermis on the leaf was 16 times greater than that fixed by isolated epidermis during a 10-min exposure. Considering my results, in light of this other work, it seems that although guard cells <u>can</u> produce the organic anions they require independently of mesophyll mediation, it appears likely that they also utilize the mesophyll as a source of reduced carbon.

More importantly, my results show that guard cells are flexible in the anion used to balance K^+, and thus help to explain the variable participation of Cl^- in stomatal regulation observed by others. As demonstrated by Figure 3B, page 20, when Cl^- is available to isolated epidermis, malate does not accumulate. Presumably, Cl^- reduces the malate content by replacing malate as a counter-ion for K^+. Alternatively, one could argue that Cl^- is, in some way, altering organic acid metabolism so that some other organic anion, rather than malate, is produced. It seems simpler, however, to postulate a direct replacement of malate by Cl. Work done on other systems supports the latter proposal: absorbable inorganic anions have been shown to replace organic acids in roots (12,23, and papers referred to in 16); an inhibition of Cl^- on ^{14}C-malate and $H^{14}CO_3$ uptake by barley and carrot root tissue has also been observed (3).

Different methods for regulating cytoplasmic pH could result in this seemingly facultative use of organic anions for osmotic regulation in guard cells. It is known that when stomates open, K^+ is taken up into the guard cells and that equivalent quantities of H^+ appear in the bathing solution of epidermal strips (27, and R. Saftner, personal communication). This ion transfer would produce an increase in OH^- inside the guard cell while, externally, the level of H^+ will rise. Guard cells must have some means for counteracting OH^- accumulation. There are at least two ways that this might be accomplished: (1) If Cl^- (or some other absorbable inorganic anion) is available, a Cl^- OH^- exchange could take place; Cl^- would then move with K^+ into the vacuole and the initial cytoplasmic pH would be maintained. (2) Organic acids could be produced with the subsequent transfer of K organate, most probably K malate, to the vacuole. The *in vitro* pH optimum for PEP carboxylase in crude extracts from *C. communis* epidermis is 8.4 (37); the rising intracellular pH might thus serve as the trigger for organic acid synthesis. In addition, the rising intracellular pH would increase the availability of HCO_3, presumably the substrate or PEP carboxylase (16). In the presence of absorbable anions, such a "trigger" would not be generated and acid synthesis would be depressed. This mechanism, which has been proposed to account for organic acid synthesis in roots during "excess" cation uptake (11), could explain how the presence of Cl^- prevents malate accumulation during stomatal opening in *V. faba*.

If this explanation is correct, one would expect to see a relation between Cl^- offered and malate formed, or aperture attained.

For example, if the uptake of Cl^- required the same energy as malate formation, then one would expect a) the stomates to attain the same aperture regardless of the quantity of Cl^- offered, and b) an inverse relation between malate formed and Cl^- supplied. If formation of organic acids required more energy than Cl^- uptake, one would expect an increase in stomatal aperture to follow an increase in external Cl^- within a certain range of Cl^- concentrations. Figure 5 (page 23) supports the latter alternative. However, the scatter in these data renders any conclusions about the relative use of Cl^- versus malate tenuous. For more definitive statements it will be necessary to measure K^+, Cl^-, and malate in the same tissue. Such measurements may also reconcile the scatter in epidermal malate content seen even in the absence of externally supplied Cl^-. The scatter in all experiments is believed to result primarily from variation in endogenous Cl^-.

When leaf sections were offered Cl^-, a decrease in epidermal malate, similar to that observed in isolated epidermis, was detected (Figure 7, page 30), although the inhibition of malate accumulation was less than that observed in isolated epidermis. Two possible explanations are: 1) increased Cl^- availability because of the fact that the epidermis was <u>in situ</u>, and 2) reduced carbon compounds could be readily obtained from the mesophyll and converted to malate.

From experiments with non-absorbable anions (27), as well as studies on fusicoccin-stimulated H^+ secretion and K^+ uptake by epidermal tissue (33, and R. Saftner, personal communication), it has been postulated that H^+-K^+ exchange is the primary event in initiating stomatal opening (22). The results of my work showing that <u>either</u>

organic or inorganic anions can be used for balancing K^+ accumulation support this proposal and tend to rule out either active uptake of Cl^- or metabolism of organic acids as the driving force behind increases in stomatal aperture.

The hypothesis previously presented for Cl^-/malate interaction based upon cytoplasmic pH can also provide an explanation for my observation that Cl^- reduces the CO_2 sensitivity of stomates. But first we must consider Rasche's general hypothesis for the effect of CO_2 on stomates (23,24,25). Raschke proposes that the response of stomates to CO_2 resides in the balance between malate synthesis in the cytoplasm and malate transfer to the vacuole. If malate is formed via carboxylation, CO_2 is required and increasing levels of CO_2 could stimulate the rate of malate formation through mass action. But an increase in malate concentration would tend to turn off further malate synthesis by feedback inhibition on PEP carboxylase. Thus, Raschke suggests that CO_2 reduces the rate of K^+ uptake by reducing the rate of organic acid formation. (In addition, acidification of the cytoplasm resulting from increased formation of 3-phosphoglyceric acid, the precursor for PEP, would tend to exclude K^+, as well as reduce the activity of the carboxylation enzymes.) Raschke also sees the possibility of a direct effect of malate on membranes. Short-chain undissociated aliphatic acids (including malate), have been shown to increase permeability in barley roots (14). The guard cell tonoplast might respond similarly to undissociated malate, leading to a net loss of K salts. This could explain how an elevation in external CO_2 could cause stomates to close.

Raschke points out that the maintenance of a high concentration of cytoplasmic malate to keep stomates closed requires consideration of malate transfer to the vacuole. This process, which is thought to require energy, probably has a certain maximum rate. In some instances, for example, at low external CO_2 concentrations, the rate of malate formation may be below the maximum rate for transport; essentially all malate formed is transferred to the vacuole and the accumulation of K salts, with resultant opening, proceeds. When stomates are induced to open from the closed state, maximum apertures are obtained not in CO_2-free air, but in air containing around 100 $\mu l\ l^{-1}$ CO_2 (5); higher amounts of CO_2 lead to narrower apertures. According to Raschke, the maximum opening is caused by a rate of malate formation which matches the maximum transport rate. Under higher CO_2 concentrations, synthesis exceeds transport causing the cytoplasmic level of malate to rise, leading to a reduction in malate synthesis and increased membrane permeability. As long as this external CO_2 concentration is maintained, the level of cytoplasmic malate will remain in equilibrium with it.

My observation of Cl^--induced reduction of CO_2 sensitivity can now be explained as a possible effect of Cl^- on cytoplasmic pH; Cl^- could circumvent the increase in cytoplasmic pH which, as previously discussed, might be the trigger for malate synthesis. Since, in the presence of Cl^-, malate synthesis is reduced, CO_2 sensitivity would also decline, and higher levels of CO_2 would be required to have a rate of malate formation exceeding malate transport. Such an explanation is consistent with the experiments on C. communis, where, when both CO_2

and Cl^- were offered, stomatal response was dependent upon their relative proportions (Figures 1 and 2, pages 15 and 17 respectively), as well as congruent with the results from V. faba in which apertures of stomates exposed to 10^{-1} eq Cl^- l^{-1} were often larger if CO_2 was also present (Table I, page 24). Experiments in which cytoplasmic pH is monitored along with uptake of K^+ and Cl^-, and malate formation are necessary to prove the validity of this hypothesis.

In the experiments on isolated epidermis of V. faba, stomates were generally more open in the absence of CO_2 than when 300 l l^{-1} CO_2 were present (Table I, page 24); their malate content was also higher (Figure 3, page 20). This indicates that epidermal strips can evolve sufficient CO_2 for malate formation. Information on the relative rates of the synthesis and transport of malate could be obtained by testing the effect of 50 or 100 µl l^{-1} CO_2 on both stomatal aperture and epidermal malate and considering these results in light of measurements on the quantity of CO_2 evolved by isolated epidermes.

When stomates close, guard cells must dispose of the osmotica they have accumulated in their vacuoles during opening. K^+, and whatever Cl^- has been utilized, move into subsidiary cells if these are present (21,26). Inorganic ions may also be held by the cell wall and/or specialized epidermal cells which seem to store the ions (24,26, 36). Results from ^{14}C labelling studies indicate that organic anions may be disposed of in three ways: 1) catabolism in the tricarboxylic acid cycle; 2) decarboxylation followed by gluconeogenesis and starch formation; and 3) release from the epidermis (6). However, it was

estimated that only a small fraction of the total malate pool was initially labelled in these studies. To determine the extent to which these three methods for disposing of organic ions are employed by guard cells, the fate of the whole malate pool must be examined. Studies with unlabelled malate cannot distinguish between the first two processes but can tell us about release from the epidermis. To this end, the experiments on ABA-induced closure were undertaken. The results support the conclusions on the basis of labelling studies: malate is released when stomates close, and moreover, release is a significant process. The fact that non-ABA-induced closure also resulted in malate release (Table I, page 24), implies that ABA is not having a specific effect on malate.

CONCLUSIONS

Stomates of epidermal strips of V. faba supplied with K^+ and a non-absorbable anion form malate as they open; the increases in malate follow a nearly linear relationship with aperture (Figure 3A, page 20). In the presence of KCl, the relation between malate and aperture breaks down (Figure 3B, page 20). Cl^- also reduces the ability of guard cells to respond to CO_2 (Table I, page 24). These effects of Cl^- on malate formation and CO_2 sensitivity have been interpreted as resulting from different methods for regulation of the cytoplasmic pH. When stomates of C. communis and V. faba are induced to close by ABA, most of the malate is released to the bathing solution (Table II, page 32 and Figure 8, page 34).

LIST OF REFERENCES

LIST OF REFERENCES

1) Allaway, W. G. 1973. Planta 110: 63-70.

2) Canvin, D. T., and H. Beevers. 1961. J. Biol. Chem. 236: 988-995.

3) Cram, W. J. 1974. J. Expt. Bot. 25: 253-268.

4) Dayanandan, P., and P. B. Kaufman. 1975. Am. J. Bot. 62: 221-231.

5) Drake, B., and K. Raschke. 1974. Plant Physiol. 53: 808-812.

6) Dittrich, P., and K. Raschke. 1977a. Submitted for publication.

7) Dittrich, P., and K. Raschke. 1977b. Submitted for publication.

8) Fischer, R. A. 1972. Aust. J. Biol. Sci. 25: 1107-1123.

9) Fischer, R. A., and T. C. Hsiao. 1968. Plant Physiol. 43: 1958-1968.

10) Goldberg, N. D., and J. V. Passonneau. 1974. Methods of Enzymatic Analysis, vol. 3 (H.V. Bergmeyer, ed.). Academic Press, New York.

11) Hiatt, A. J. 1967. Z. Pflanzenphysiol. 56: 233-245.

12) Hiatt, A. J., and S. B. Hendricks. 1967. Z. Pflanzenphysiol. 56: 220-232.

13) Humble, G. D., and K. Raschke. 1971. Plant Physiol. 48: 442-453.

14) Jackson, P. C., and J. M. Taylor. 1970. Plant Physiol. 46: 538-542.

15) Nishida, K. 1963. Physiol. Pl. 16: 284-298.

16) Osmond, C. B. 1976. Transport in Plants, vol. 2, pt. A (U. Lüttge and M. G. Pitman, eds.). Springer-Verlag, Berlin-Heidelberg.

17) Outlaw, W. H., Jr. and O. H. Lowry. 1976. Plant Physiol. 57: Supplement 43, #225.

18) Pallaghy, C. K., and R. A. Fischer. 1974. Z. Pflanzenphysiol. 71: 332-344.

19) Pallas, J. E. Jr., and B. G. Wright. 1973. Plant Physiol. 51: 588-590.

20) Pearson, C. J., and F. L. Milthorpe. 1974. Aust. J. Pl. Phys. 1: 221-236.

21) Penny, M. G., L. S. Kelday, and D. J. F. Bowling. 1976. Planta 130: 291-294.

22) Raschke, K. 1976. Transport and Transfer Processes in Plants (I. F. Wardlow, J. B. Passioura, eds.), Academic Press, New York.

23) Raschke, K. 1975. Planta 125: 234-259.

24) Raschke, K. 1975. Ann. Rev. Plant Physiol. 26: 309-340.

25) Raschke, K., and P. Dittrich. 1977. Submitted for publication.

26) Raschke, K., and M. P. Fellows. 1971. Planta 101: 296-316.

27) Raschke, K., and G. D. Humble. 1973. Planta 115: 47-57.

28) Raschke, K., and M. P. Pierce. 1975. Plant Research '74, MSU/AEC Plant Res. Lab., Mich. State Univ., 31.

29) Sawhney, B. L., and I. Zelitch. 1969. Plant Physiol. 44: 1350-1354.

30) Shaw, M., and G. A. Maclachlan. 1954. Can. J. Bot. 32: 784-794.

31) Shenk, G. H. 1973. Absorption of Light and UV Radiation: Fluorescence and Phosphorescence Emission. Allyn and Bacon, Inc., Boston. pp. 147-181.

32) Tucker, D. J., and T. A. Mansfield. 1971. Planta 98: 157-163.

33) Turner, N. C. 1973. Am. J. Bot. 60: 717-725.

34) Ulrich, A. 1941. Am. J. Bot. 28: 526-537.

35) Willmer, C. M., and P. Dittrich. 1974. Planta 117: 123-132.

36) Willmer, C. M., and J. E. Pallas, Jr. 1973. Can. J. Bot. 51: 37-42.

37) Willmer, C. M., J. E. Pallas, Jr., and C. C. Black, Jr. 1973. Plant Physiol. 52: 448-452.

38) Witt, I. 1974. Methods of Enzymatic Analysis, vol. 3 (H. V. Bergmeyer, ed.) Academic Press, New York.