STUDIES ON FACTORS CONTROLLING OPENING OF STOMATA IN THE CAM PLANT *CRASSULA ARGENTEA*

By

MARK VERNON LOUCKS

Bachelor of Science

Oklahoma State University

Stillwater, Oklahoma

1976

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
MASTER OF SCIENCE
May, 1979

Name: Mark Vernon Loucks Date of Degree: May, 1979

Institution: Oklahoma State University Location: Stillwater, Oklahoma

Title of Study: STUDIES ON FACTORS CONTROLLING OPENING OF STOMATA IN THE CAM PLANT CRASSULA ARGENTEA

Pages of Study: 35 Candidate for Degree of Master of Science

Major Field: Botany

Scope and Method of Study: The effect of KCl, K_2SO_4, pH, abscisic acid (ABA) and N,N'-dicyclohexylcarbodiimide (DCCD) on stomatal behavior were studied in the crassulacean acid metabolism (CAM) plant Crassula argentea. Epidermal strips were made from the leaves of intact plants and floated on the treatment solutions. The strips were then examined under a microscope and the stomatal aperture measured. K^+-specific stains were made of acid-treated strips and of ABA and DCCD treated strips to ascertain potassium fluxes. Characterization of epidermal strip nitrophenylphosphatase (NPPase) activity was also undertaken. The K_m of epidermal NPPase for nitrophenylphosphate (NPP), and the effects of K^+, pH, ABA and DCCD on NPPase activity was examined. The relationship between stomatal behavior in C. argentea and NPPase activity was then examined.

Findings and Conclusions: Both KCl and K_2SO_4 stimulated stomatal opening in C. argentea with an optimal concentration of 100 mM K^+. Low pH induced the opening of stomata; this low pH response was abolished by ABA and DCCD. The acid-induced opening was associated with K^+ movement into the guard cells as shown by K^+-specific staining. Potassium was localized in the subsidiary cells of epidermal strips treated with ABA. In the epidermal strips treated with DCCD, K^+ was found primarily in intercellular spaces. The K_m of C. argentea NPPase activity was 4 mM. In the dark, low pH stimulated NPPase activity, whereas high pH inhibited it. The NPPase activity was stimulated by potassium and inhibited by ABA and DCCD. These results are consistent with the hypothesis that stomatal opening in C. argentea is induced and enhanced by low pH, and that this opening response is regulated by a guard cell membrane-bound ATPase which actively takes up K^+ from the surrounding subsidiary cells.

ADVISER'S APPROVAL James D Ownby

STUDIES ON FACTORS CONTROLLING OPENING OF

STOMATA IN THE CAM PLANT

CRASSULA ARGENTEA

Thesis Approved:

James D Ownby
Thesis Adviser

Glenn W. Todd

Paul E. Richardson

Norman N Durham
Dean of the Graduate College

ACKNOWLEDGMENTS

I wish to extend my most sincere appreciation to Dr. James D. Ownby for his inspiration, moral support, and valuable time and assistance in completing this research and my graduate study. I thank him as well for his invaluable criticisms and aid in preparing this manuscript.

I would also like to thank Dr. Glenn W. Todd and Dr. Paul Richardson for their contributions and time spent on my committee.

I would also like to thank Dr. Edward Grula and Dr. Eddie Basler for their gifts of DCCD.

I wish to express my appreciation to my parents, Mr. and Mrs. Keith W. Loucks. Without their love and financial support, entering graduate college would have been impossible.

Finally, I wish to express my love and appreciation to my wife, Connie, who suffered through much trial and tribulation to see the denouement of this study. Without her support and loving devotion, the attainment of this degree would have been an empty accomplishment.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	MATERIALS AND METHODS	8
III.	RESULTS	11
	Effect of KCl and K_2SO_4	11
	Effect of pH	11
	Effect of ABA and DCCD	14
	Characterization of Nitrophenylphosphatase Activity in Epidermal Strips	17
IV.	DISCUSSION	26
LITERATURE CITED		31

LIST OF FIGURES

Figure		Page
1.	Effects of KCl Concentration on Stomatal Width	12
2.	Effects of Varying Concentrations of KCl and K_2SO_4 on Stomatal Width	13
3.	Effects of pH on Stomatal Aperture	15
4.	Titratable Acidity and Stomatal Aperture During the Transition From the Light to the Dark Period	16
5.	View of Stomatal Apparatus From an Epidermal Strip Incubated in the Dark for 4 hr at pH 3.5	17
6.	Treatment Identical With That of Figure 5 Except That the Test Solution Contained 10 uM ABA	17
7.	Localization of K^+ in Epidermal Strips Incubated in the Dark at pH 3.5 in the Presence of 50 uM DCCD, x80	19
8.	Localization of K^+ in Epidermal Strips Incubated in the Dark at pH 3.5 in the Presence of 50 uM DCCD, x500	19
9.	Effect of Substrate Concentration on NPPase Activity in Epidermal Strips From C. argentea	20
10.	Effect of pH on NPPase Activity in Epidermal Strips of C. argentea	21
11.	NPPase Activity as a Function of Time at pH 4.5 and pH 5.5	22
12.	Effect of DCCD and ABA on NPPase Activity	24
13.	Effects of K^+ on NPPase Activity at pH 7.5	25

ABBREVIATIONS

ABA	abscisic acid
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CAM	Crassulacean Acid Metabolism
DCCD	n, n'-dicyclohexylcarbodiimide
EDTA	ethylenediaminetetraacetric acid
NP	nitrophenol
NPP	nitrophenylphosphate
NPPase	nitrophenylphosphatase

CHAPTER I

INTRODUCTION

The stomata of land plants are the primary site of gas exchange and transpirational water loss in plants. The physiology and regulation of their movements, opening and closing, is thus an area of particular concern in plant physiology. Stomates respond to a variety of environmental parameters. Their opening appears to be regulated primarily by internal CO_2 concentration and tissue water content. Fischer (1968) showed that epidermal strips of Vicia faba exposed to CO_2-free air opened in the dark. Research with Zea mays also showed that the guard cells regulated their opening and closing in response to CO_2 concentration. Reduction of CO_2 caused a rapid opening response, while increased levels induced closure (Raschke, 1972). Intercellular levels of CO_2 remained fairly constant due to the opening response of Z. mays stomata (Raschke, 1970, 1972). Use of CO_2 analogues such as ethylene, albene or SO_2 showed that the response by guard cells was specific for CO_2 (Pallaghy and Raschke, 1472; Majernick and Mansfield, 1972). Environmental parameters such as light that influence photosynthesis, also influence stomatal movement. Light causes opening in most species indirectly by causing a photosynthetic reduction of intercellular CO_2 in leaves (Raschke, 1975). The exact triggering mechanism for CO_2 control of stomatal movement is unknown at present (Fischer, 1968).

The other critical regulator of stomatal movements is the water content of the plant. Well-watered plants can become insensitive to CO_2 levels, and their stomata remain open (Raschke, 1975). Stomata of water-stressed plants remain open until a deficit threshold is reached, then they close very rapidly (Hsiao, 1973). This threshold water potential varies in the range -7 to -18 atmospheres (Hsiao,1973). Isolated epidermal strips did not show this threshold. In water-deprived leaves, the stomata closed only after a delay of some minutes, then did not reopen quickly after watering (Stalfelt, 1929; Raschke, 1975). This suggested an active metabolic mechanism; it was observed that abscisic acid (ABA) levels increase substantially and rapidly in water-stressed leaves (leaf water potential $\leq$ -10 atm, average of studied species) (Zeevaart, 1971; Most, 1971; Loveys and Kriedemann, 1973; Horn and Wright, 1972). ABA is an inhibitor of stomatal opening and a doubling of ABA content in wilting leaves brings on stomatal closure (Kriedemann et al., 1972). This has been called the hydroactive control of stomatal movement by Raschke (1975).

The exact biochemical mechanism behind ABA control of stomatal opening is unknown. It is thought to inhibit cyclic photophosphorylation of guard cell chloroplasts, thus preventing any active uptake of ions necessary for opening. ABA may also act to block organic acid formation in guard cells (Ragavendra, Rao, and Das, 1976).

The physiological events that led to opening are designed to decrease the water potential of the guard cells, causing an influx of water, and allowing the two guard cells to swell apart, effecting the creation of the stomatal aperture (Aylor, Parlange and Krikorian, 1973).

The guard cells have a thickened wall next to the pore, which, combined with radial micellation, causes increased turgor to form the aperture (Aylor, Parlange and Krikorian, 1973). It is the radial miscellation of the guard cells which is primarily responsible for pore formation, but the surrounding epidermal cells and especially the subsidiary cells play a part as well. They have a "mechanical advantage" over the guard cells (Demichele and Sharpe, 1973); this is manifested in the hydropassive feedback loop: as turgor of the leaf epidermis increases in a well-watered plant, the surrounding cells tend to push against the guard cells and keep them closed or slightly less open; in a wilting leaf, the epidermal cells become flaccid faster than the guard cells, and a transient increase in opening occurs (Demichele and Sharpe, 1973; Raschke, 1975).

It is the change in osmotic potential of the guard cells which ultimately causes stomatal movement. Earlier theories advanced by Scarth and others suggested that the decrease in osmotic potential prior to opening was induced by photosynthetic reduction of CO_2 in the guard cells (guard cells are the only cells in the epidermis which contain chloroplasts, Essau, 1965). This resulted in an increased pH which activated starch phosphorylase, the enzyme which hydrolyzes starch to sugar, and thus decreases water potential (Scarth, 1932; Salisbury and Ross, 1978). Experimental evidence later showed that if such a conversion took place, it was too slow and of too small a magnitude to account for the rapid opening observed (Raschke, 1975).

Immamura (1943) described experiments implicating the involvement of potassium in stomatal opening. Twenty years later, Immamura's work was "discovered," and a flood of papers revealed that the K^+ influx

into guard cells was large and rapid enough to account for at least most of the decrease in osmotic potential (Fujno, 1967; Fischer and Hsiao, 1968; Raschke, 1975). The influx of potassium has been observed in all species studied, including pteridophytes, gymnosperms, and crassulacean acid metabolism (CAM) plants (Willmer and Pallas, 1973; Dayanandan and Kaufman, 1975).

Associated with the potassium ion influx are several charge-balancing ionic movements. As K^+ moves into the guard cells, hydrogen ions move out (Raschke and Humble, 1973), chloride ions move in (Schnable and Ziegler, 1977), and organic acids accumulate (Allaway, 1973) within the guard cells, or possibly are transported in from adjacent cells (Bowling, 1976). All these events occur in the species which have been studied, but their relative importance varies. For example, in corn, Cl^- influx accounts for 40% of the positive K^+ charges, whereas in Vicia faba, it counterbalances less than 20% of the potassium content of guard cells (Raschke, 1975; Pallaghy and Fischer, 1974). The balance is made up by H^+ extrusion and the transport or accumulation of malic acid, aspartate and citrate (Raschke, 1975). Allaway (1972) observed that malate accumulation in V. faba guard cells accounted for half of the potassium charge.

The ion fluxes described above all act to create a water potential gradient for the flow of water into or out of guard cells. However, all of the species mentioned, and those used for nearly all studies of stomatal physiology, are the typical C-3 and C-4 plants with day-time opening of their stomata. The stomata of plants exhibiting crassulacean acid metabolism (CAM), on the other hand, open their stomata at night rather than during the day, reducing transpirational water losses

considerably (Noggle and Fritz, 1976). CAM plants are adapted to arid habitats, and are found in the deserts and steppes of the world, also as epiphytes in the tropics and subtropics. The more xeric the habitat, the more prevalent are CAM plants (Osmond, 1978).

There are several factors which are characteristic of CAM plants (see Black and Williams, 1976). Besides an inverse stomatal rhythm, CAM plants show marked diurnal fluctuations in the organic acid content, particularly malic acid, of the green tissue (Goh et al., 1977; Kluge, 1976; McWilliams, 1970; Nishida, 1963; Pucher et al., 1947). During the night, malate levels increase up to twice the daytime amount and decrease the tissue pH markedly (Osmond, 1978). This is followed by an increase in tissue pH in conjunction with a decrease in malic acid content in the hours between sunrise and sunset. Perhaps the most characteristic feature of CAM is the net dark fixation of CO_2 by the leaves (Black and Williams, 1976). The starch content of CAM leaves decreases at night and increases during the day. Finally, CAM plants invariably have succulent green tissue (Osmond, 1978).

At night, CAM plants fix CO_2 by combining it with phosphoenolpyruvate (PEP) to form oxaloacetate (OAA) in the presence of PEP carboxylase. Malic acid is then formed from the OAA by the action of malate dehydrogenase (Kluge, 1976). The malate is transported into the leaf cell vacuole and stored there. According to the model of Kluge, when a critical turgor is reached by the swelling vacuole, the net influx of malate switches to a net efflux. This generally happens after dawn. The released malate is then broken down into CO_2 and pyruvate, and the CO_2 is used by the chloroplasts to form sugars by the Calvin-Benson cycle. The regulation of CAM is a subject of controversy, complicated

by the observation that some CAM species are obligate and others are facultative. Obligate CAM plants show nocturnal stomatal opening and CO_2 fixation regardless of environmental conditions, whereas facultative plants revert to typical C-3 day CO_2 fixation when adequately watered (Hartsock and Nobel, 1976; Crews et al., 1976). Any research using CAM plants must take this fact into account.

Of interest in this study was the rhythm of nocturnal opening in CAM plants. What are the differences on a physiological level that allow CAM stomata to open at night and close during the day? Most workers in this area view it simply as a response to CO_2 levels (Larcher, 1973). During the day, when the intercellular spaces are thought to be flooded with CO_2 released from malate, the stomata close. At night malate levels, and thus CO_2 levels, are severely reduced, inducing the stomata to open. However, according to Kluge's model (1976), once fixed as malate, CO_2 does not leave the mesophyll cells. If indeed CAM stomata are responding to CO_2 levels, the means by which the mesophyll communicates with the guard cells remains a mystery. One possible explanation concerns the involvement of a membrane-bound ATPase in the guard cell.

Several lines of evidence point to the possibility that the uptake of potassium ions by guard cells is active and regulated by membrane-bound ATPases. Membrane-bound ATPases involved in ion movement have been found in many higher plant systems (Lai and Thompson, 1971; Hodges et al., 1972; Hodges, 1976). Thomas (1970), working with tobacco and broadbean epidermal strips, detected the presence of an ouabain-sensitive enzyme involved in stomatal movement. Ouabain is a known inhibitor of animal ATPases, and when added to Thomas's experimental medium, it inhibited stomatal opening. Other experiments showed that

addition of ATP in the dark and ADP in the light stimulated the opening of tobacco stomata (Thomas, 1971).

In Commelina benghalensis the involvement of two ATPase isozymes in stomatal movement has been postulated (Raghavendra, Rao and Das, 1976). During the day when the stomata are open, an ATPase with a pH optimum of pH 7.5 was extracted from C. benghalensis epidermal strips; this isozyme was linked with stomatal opening (C. benghalensis is a C-3 plant). Potassium chloride enhanced the activity of this isozyme. An isozyme, thought to be associated with stomatal closure, with a pH optimum of pH 5.5, was extracted from epidermal strips of plants in the dark. This isozyme was stimulated by calcium. Raghavendra, Rao, and Das, using histochemical stains, also found the greatest ATPase activity of epidermal strips to be localized in guard cells.

The investigation of ATPase involvement in CAM stomatal movement was part of the research for this study. Crassula argentea, or jade plant, was chosen because of its availability, ease of maintenance, and because its epidermis could be easily stripped from its leaves. The influence of K^+, in association with Cl^- and SO_4^{-2} was studied. The effects of ABA on stomatal opening and ATPase were also studied. DCCD, a known inhibitor of cation-requiring membrane ATPases in higher plants and animals, was also used to study ATPase involvement (Leonard and Hodges, 1973). Answers were sought to these questions as well: (1) Is C. argentea an obligate or a facultative CAM plant? (2) Where is the K^+ localized in inhibitor and acid-treated epidermal strips? (3) What is the effect of pH on stomatal opening and potassium movement in the jade plant? (4) What are the pH optima and other characteristics of the epidermal ATPases of jade plant?

CHAPTER II

MATERIALS AND METHODS

Crassula argentea was purchased at commercial greenhouses and kept in a Sherer growth chamber under an 11 hr photoperiod with 25 C day and 15 C night temperatures. Total irradiance was 240 uEinsteins $m^{-2}sec,^{-1}$ which was supplied by 8 Sylvania cool white fluorescent tubes supplemented with four 25-W incandescent bulbs. Plants used in the experiments were watered once a week with 50 ml of tap water.

Epidermal strips for experimentation were prepared using freshly harvested leaves by breaking the tip of the youngest mature leaves from the adaxial surface and drawing the broken section over the abaxial surface. The resulting epidermal peel was then sectioned to 0.5 cm^2, washed briefly in distilled water at room temperature, and placed in the experimental solutions.

For experiments involving exogenous K^+, solutions of 10, 50, 100, and 250 mM KCl or K_2SO_4 were prepared. The epidermal strips were floated on the solutions with their cuticle side up. The effects of pH on stomatal movement were determined by floating strips on 10mM KCl adjusted to the desired range of pH (3 to 8) using 0.1 M HCl or 0.1 M NaOH. The solutions were aerated with air passed through four saturated solutions of $Ca(OH)_2$ to reduce CO_2 concentration to less than 10 ppm as determined by a Beckman Model 15A infrared analyzer.

The incubation period for these experiments was 4 hr. Petri dishes were wrapped in two layers of aluminum foil for the dark treatment. After incubation, the strips were placed on microscope slides and examined at 400x using a Nikon phase contrast microscope. Twenty stomata were randomly chosen and measured at the widest point of their pores with a calibrated micrometer.

Inhibitor studies were conducted by adding 10 uM ABA or 50 uM DCCD (obtained from K & K Laboratories, Plainview, N.Y.) to the low pH solution.

Leaf acidity was measured by titrating leaf extracts to neutrality using 0.1N NaOH (Szarek and Troughton, 1976). Leaf extracts were obtained by harvesting leaves at set intervals, freezing them, then grinding them with a Waring blender in distilled water. The resulting brei was filtered through Whatman #1 filter paper in a buchner funnel, and the filtrate used for measurement of leaf acidity.

Sodium cobaltinitrite was used to stain for K^+, after the method of Macallum (1905) as modified by Dayanandan and Kaufman (1975). Strips were floated on cold 20mM $CaCl_2$ for two minutes, rinsed in cold distilled water, and placed in 20% sodium cobaltinitrite stain for 10 minutes. After being rinsed in distilled water, the strips were placed in 5% ammonium sulfide with 1% Tween 20 for 2 minutes. This caused the formation of dark cobalt sulfide precipitate wherever K^+ had been located. The strips were then examined on microscope slides at 400x using a Nikon phase contrast microscope.

In order to study possible involvement of ATPase in C. argentea stomatal movements, NPP, a high energy pseudosubstrate for ATPases, was used. The Km studies were conducted using 0, 2, 4, 6, and 8 mM NPP.

All other studies were performed with 4 mM NPP. Incubation solutions without potassium included 0.5 M Tris maleate, adjusted to the desired pH with 1 M NaOH (pHs used were 4.5, 5, 5.5, 6.5, 7.5, 8.5), 2 mM $MgCl_2$ and 2 mM EDTA. Potassium-containing solutions were made containing 0, 10, 25, 50, 100, 250 mM KCl and all the above, except $MgCl_2$. The pH of K^+-containing solutions was pH 7.5.

After incubating the strips in the above solutions for 1 hr, the epidermal strips were removed, weighed, and discarded. The pH of the solutions was adjusted to pH 7.5 using 1 M NaOH and their absorbance measured with a Bauch & Lomb Spectronic 20 or a Perkin-Elmer model 202 spectrophotometer at wavelength 400 nm. The amount of phosphate released was then calculated, using the extinction coefficient, a = 0.148 $mM^{-1}cm^{-1}$ (Lang, 1962), for nitrophenol.

CHAPTER III

RESULTS

Effect of KCl and K_2SO_4

Both KCl and K_2SO_4 stimulated stomatal opening in the dark. At lower concentrations (0-10 mM) of KCl, light inhibited the opening response. The optimum concentration for opening was 100 mM for KCl and 50 mM for K_2SO_4, which corresponds to a 100 mM K^+ ion concentration in both cases. These results are shown in Figures 1 and 2. The stock solutions used for these experiments were slightly acid at pH 6.5. In all cases, higher concentrations of K^+ ($\geq$ 250 mM) were less stimulatory than the 100 mM K^+ solutions.

Preliminary experiments (data not shown) also demonstrated that NaCl in no case stimulated stomatal movement, opening or closing, in the light or the dark.

Effect of pH

One problem encountered in the study of epidermal strips was the wide variability in response: closed stomata were often observed adjacent to completely open stomata. Only those epidermal strips which appeared fairly uniform in their response were used to measure stomatal aperture. Fischer (1968) noted similar problems with broad-bean. The pH response was tested using unbuffered distilled water

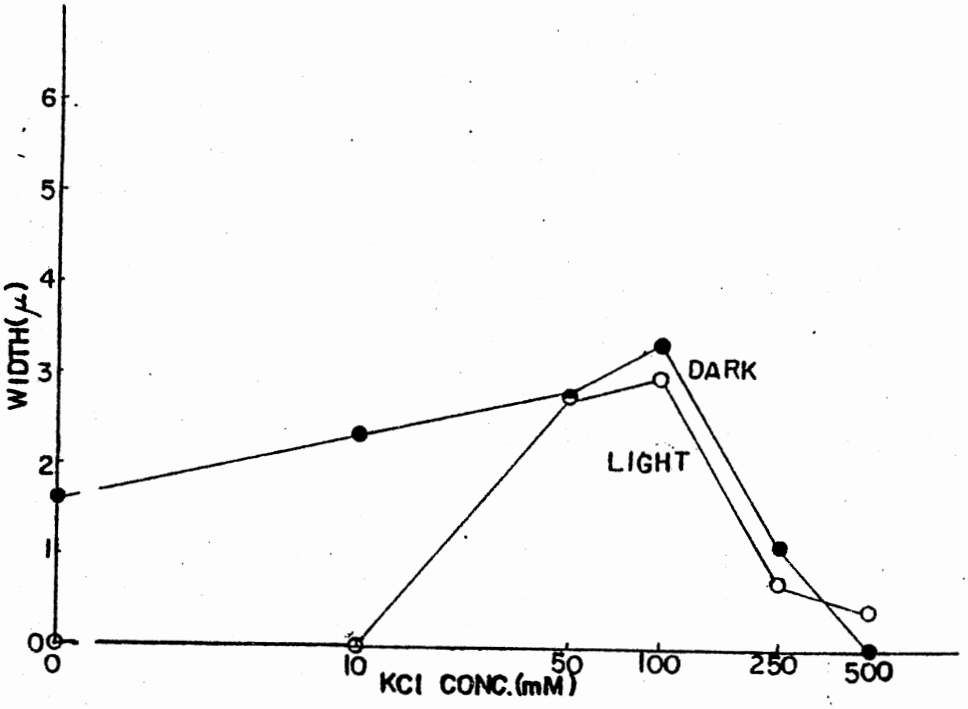

Figure 1. Effects of KCl Concentration on Stomatal Width. Epidermal strips were incubated for 4 hours either in the light (open circles) or the dark (closed circles). A typical experiment is shown here.

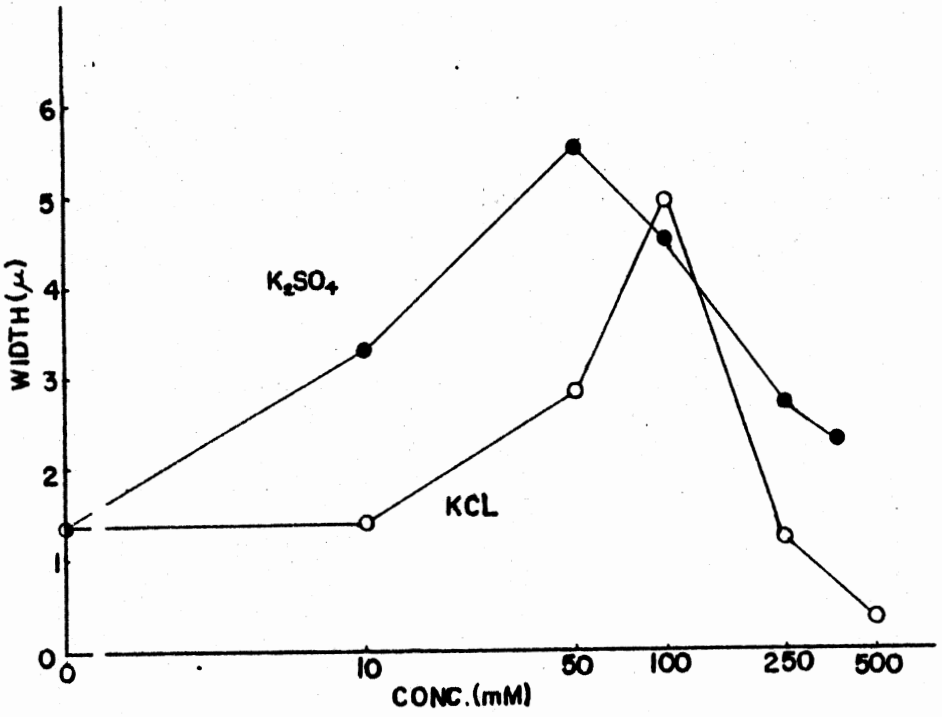

Figure 2. Effects of Varying Concentrations of KCl and K_2SO_4 on Stomatal Width. Epidermal strips were taken from plants in the middle of the light period and incubated for 4 hours in the dark. The data shown represent the results of a typical experiment. o = KCl, ● = K_2SO_4

acidified with HCl. In all cases, low pH (pH 4 to 5) stimulated an opening response. Higher pHs (pH 7 to 8) inhibited opening (Figure 3).

The nocturnal opening of stomata and subsequent accumulation of acid in the leaf, two characteristics of CAM plants, are shown for C. argentea in Figure 4. The titratable acidity increased dramatically during the night. The stomata, closed during the day, were fully open two hours after lights out.

Preliminary experiments comparing a well-watered plant with a water-stressed specimen showed no appreciable differences in the titratable acidity between the two treatments. This indicated that jade plant is an obligate CAM plant (see Introduction).

Acid treated epidermal strips were stained with the K^+-specific stain, sodium cobaltinitrite, which leaves deposits of insoluble cobalt sulfide wherever K^+ is located in the strips (Macallum, 1905). The stain showed that the acid-induced opening is associated with a K^+ influx into the guard cells (Figure 5). It was important to show the association of K^+ influx to the guard cells and the opening response at low pH, since there was the possibility that the opening was induced by acid damage to the cells. Since there was a K^+ influx to the guard cells, this appears not to be the case.

Effect of ABA and DCCD

ABA inhibited the opening response in the acid solutions which normally induced opening. Potassium-specific staining of ABA-treated strips, incubated at low pH, showed localization of K^+ in the subsidiary cells (Figure 6).

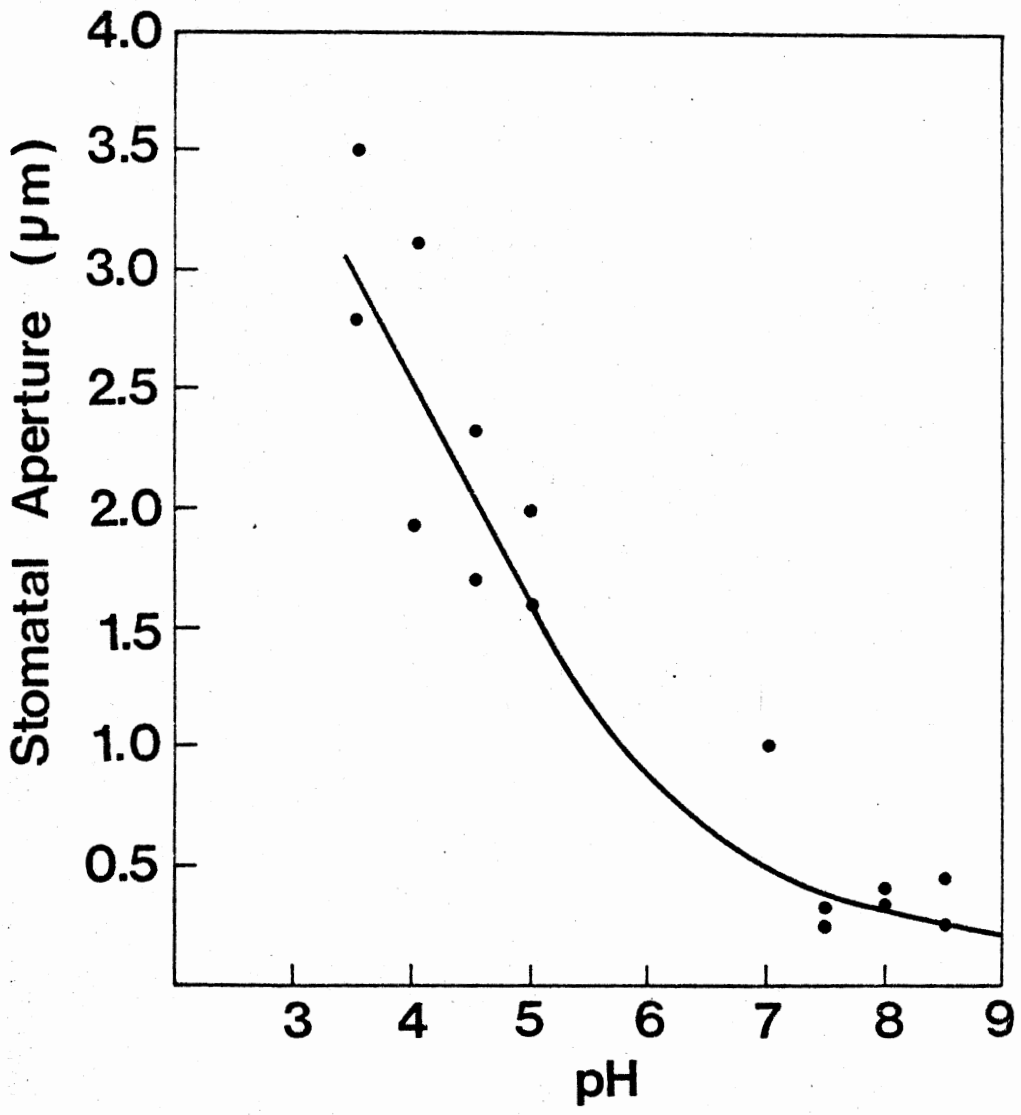

Figure 3. Effects of pH on Stomatal Aperture. Epidermal strips were floated on 10 mM KCl, cuticle up, for 4 hours in the dark. The sealed flasks containing the strips were aerated with air in which the CO_2 concentration was less than 10 ppm.

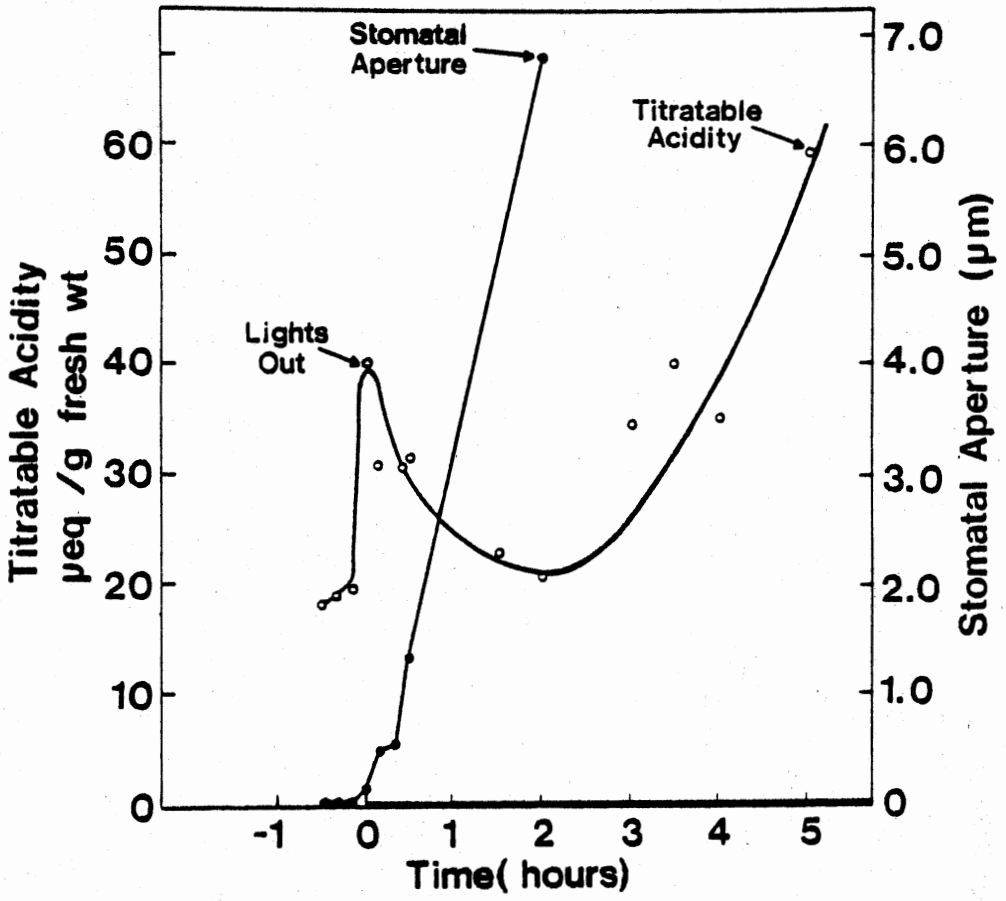

Figure 4. Titratable Acidity and Stomatal Aperture During the Transition From the Light to the Dark Period. The data represent the results of four experiments.

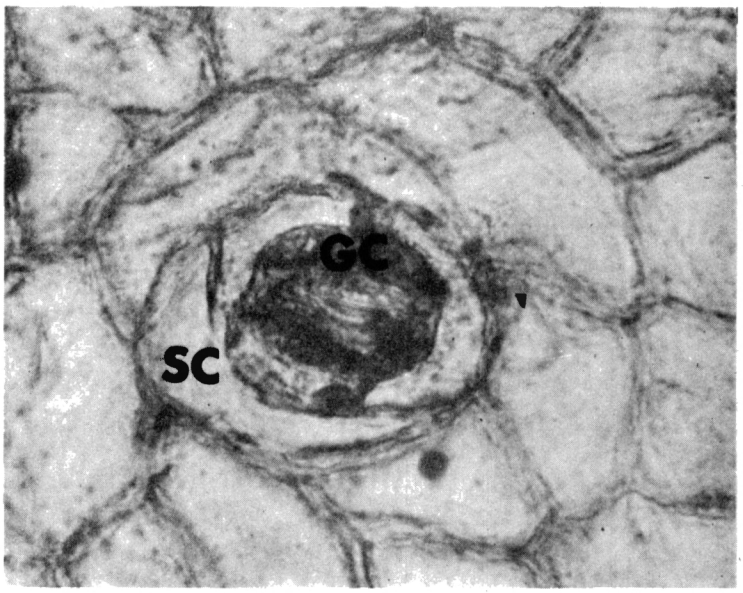

Figure 5. View of Stomatal Apparatus From an Epidermal Strip Incubated in the Dark for 4 hr at pH 3.5, x400. GC, guard cell; SC, subsidiary

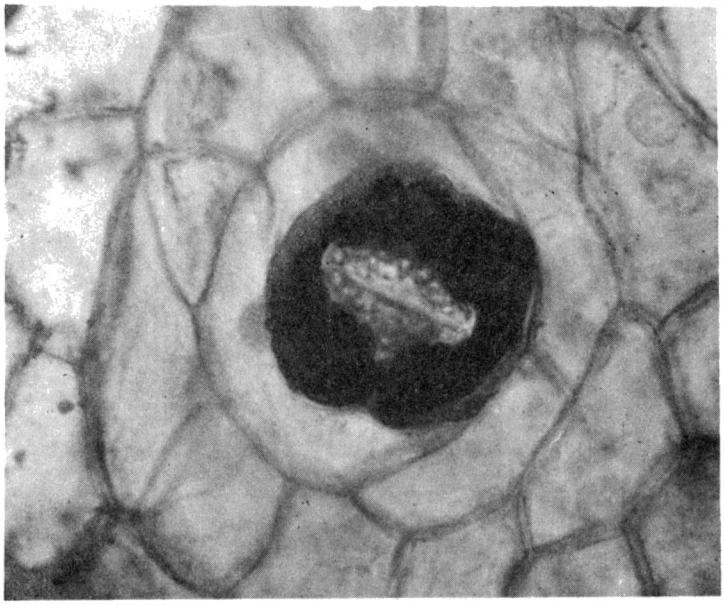

Figure 6. Treatment Identical With That of Figure 5 Except That the Test Solution Contained 10 uM ABA, x400

DCCD also acted to inhibit the opening response of strips floated in acid pH solutions. Strips stained with sodium cobaltinitrite after treatment with DCCD and acid pH showed localization of K^+ primarily in the intercellular species as determined by optical sectioning (Figures 7 and 8). Neither the guard cells nor the subsidiary cells appeared to contain high concentrations of K^+ under these experimental conditions.

Characterization of Nitrophenylphosphatase Activity in Epidermal Strips

Experiments with nitrophenylphosphate (NPP), using epidermal strips varying in weight from 0.5 mg to 84.0 mg, showed that tissues between one and ten mg gave the most consistent values without exhausting the substrate. Kinetic studies were carried out in which the amount of nitrophenol released per hour per mg fresh weight was determined as a function of NPP concentration. The Km estimated from this experiment was 4 mM, which corresponds well with data obtained from animal tissues (Albers and Koval, 1972) (Figure 9). For subsequent characterization of NPPase activity, the tris-maleate buffer was made to 4 mM NPP and adjusted to the desired pH (see Materials and Methods).

A comparison of um NP released h^{-1} mg^{-1} fresh weight versus pH (range pH 4.5 to pH 8.5) in epidermal strips taken from leaves one hour after the onset of darkness showed the greatest NPPase activity at low pH with approximately 80% lower activity at pH 8.5 (Figure 10). A time study at pH 5.5 revealed that NPPase activity increases for the first hour after the onset of darkness and leveled off about 2 to 3 hours into the night (Figure 11). A similar study at pH 4.5 revealed increased NPPase activity just prior to darkness.

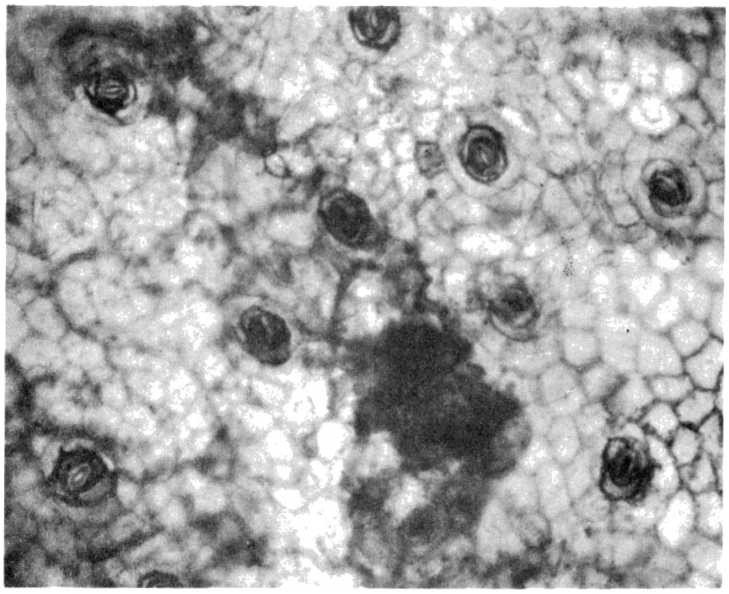

Figure 7. Localization of K^+ in Epidermal Strips Incubated in the Dark at pH 3.5 in the Presence of 50 uM DCCD, x80

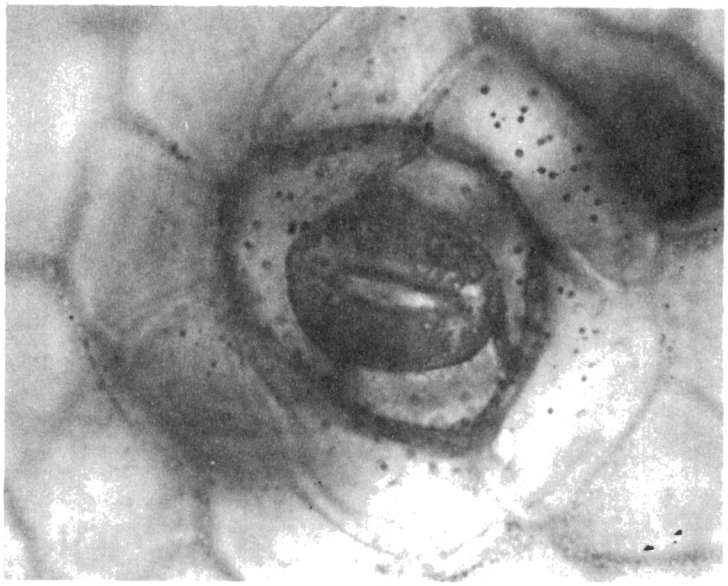

Figure 8. Localization of K^+ in Epidermal Strips Incubated in the Dark at pH 3.5 in the Presence of 50 uM DCCD, x500

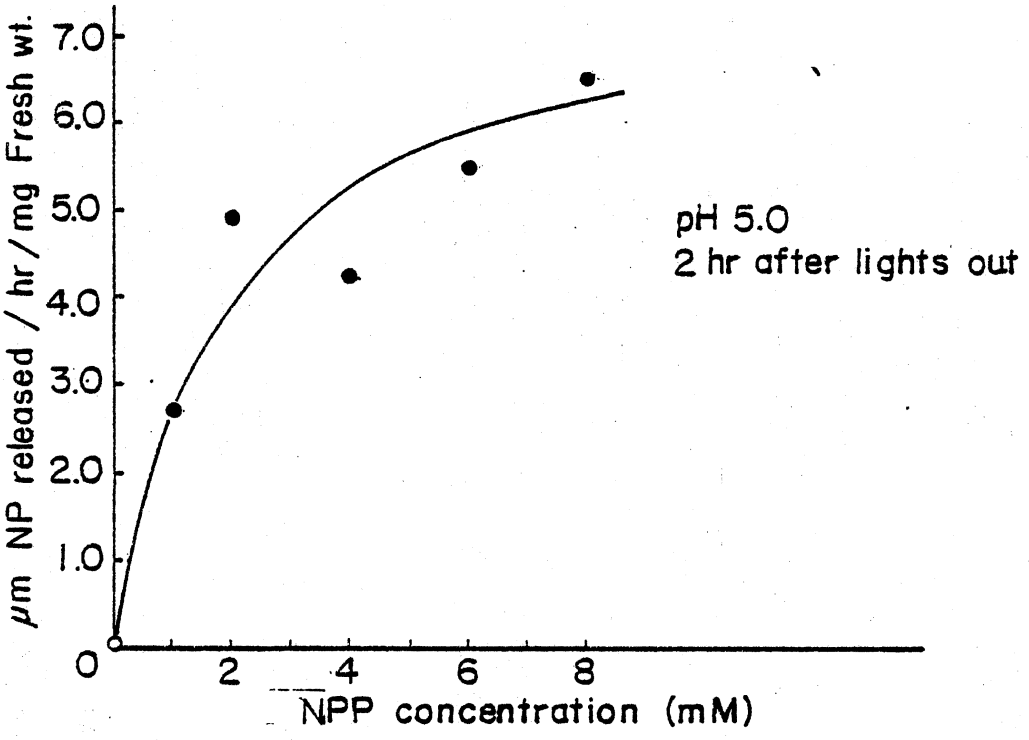

Figure 9. Effect of Substrate Concentration on NPPase Activity in Epidermal Strips From C. argentea. The strips were floated in the appropriate solutions in foil-wrapped petri dishes. All solutions had a pH of 5.0. The strips were taken from plants two hours into the night. The results of a typical experiment are shown here.

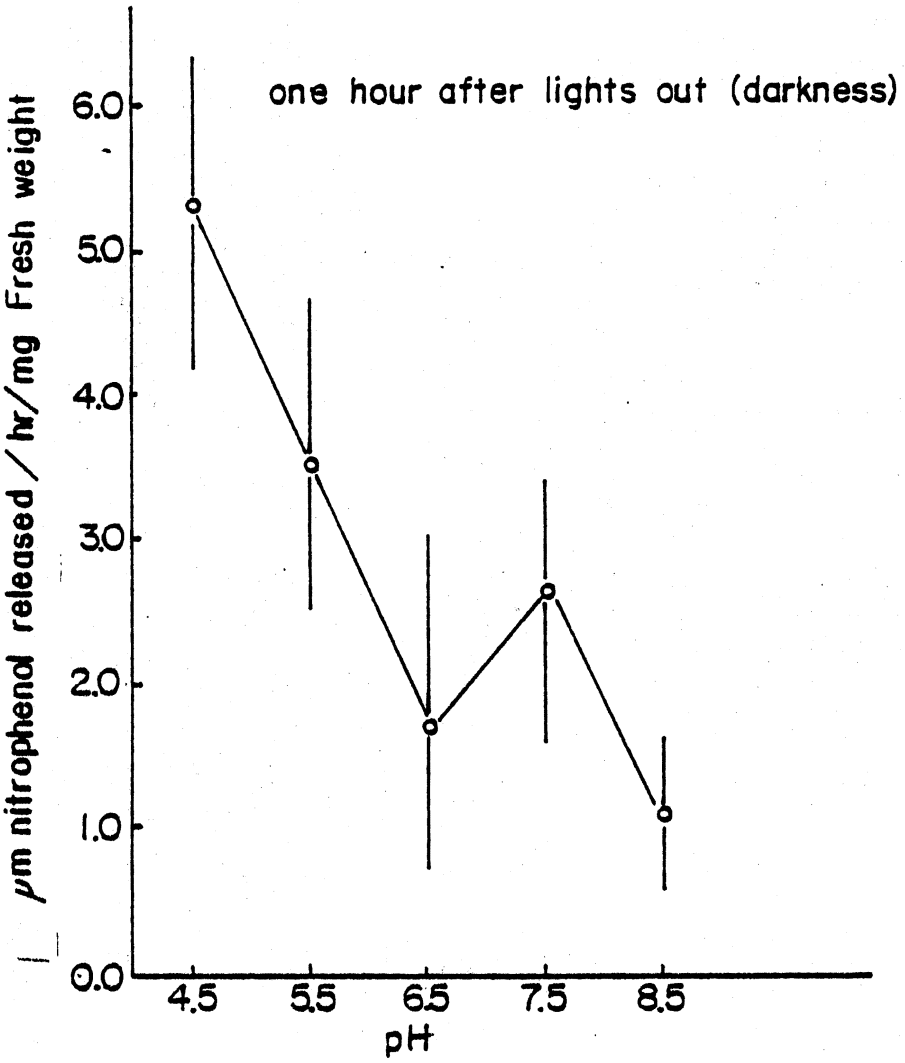

Figure 10. Effect of pH on NPPase Activity in Epidermal Strips of C. argentea. Epidermal strips were floated on the appropriate solutions in foil-wrapped petri dishes for 1 hr. The strips were taken from the leaves of plants one hour into the night. The vertical lines represent the standard deviation of the results from 4 experiments.

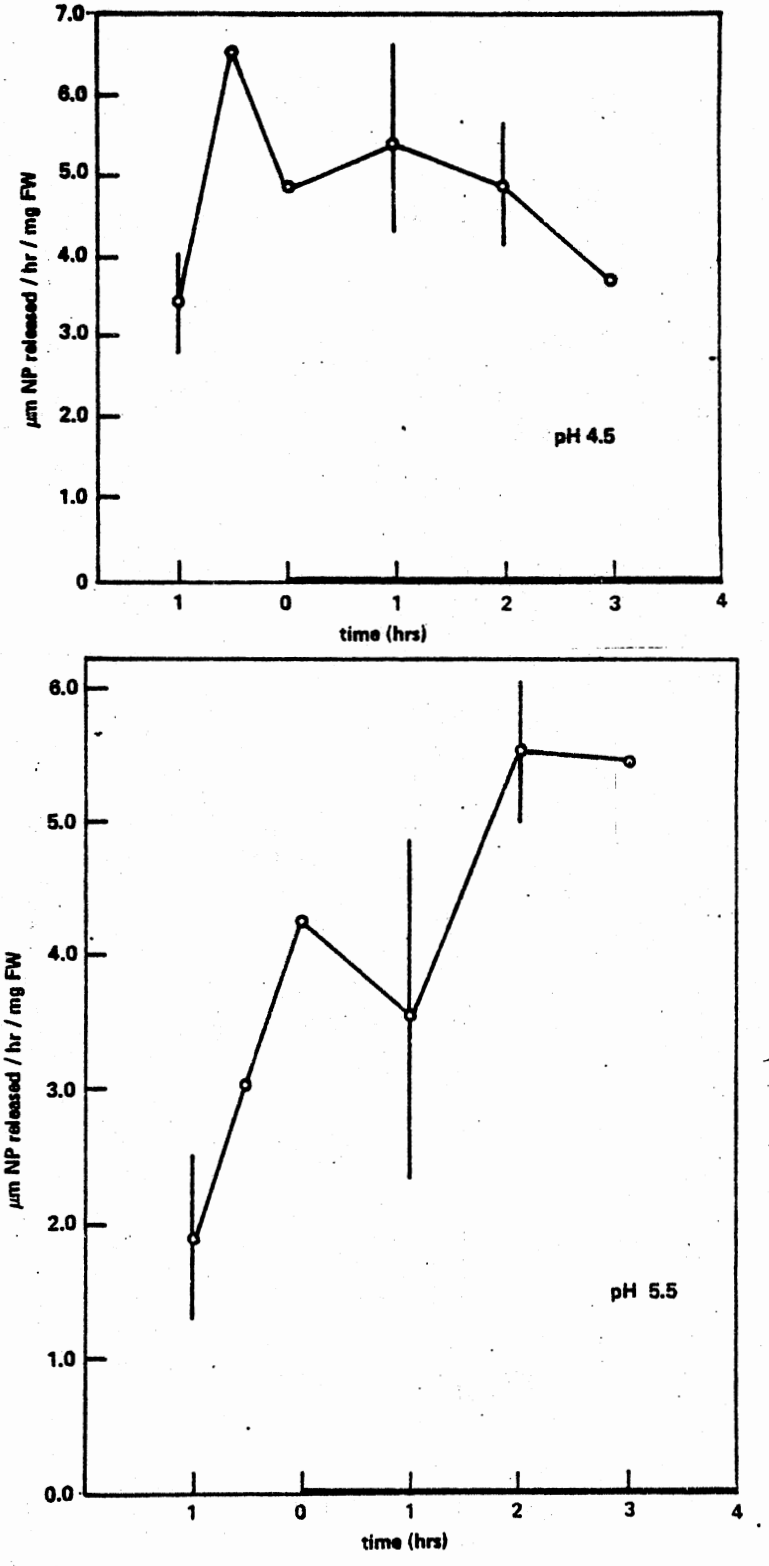

Figure 11. NPPase Activity as a Function of Time at pH 4.5 and ph 5.5. The vertical lines show the standard deviation of results from 4 experiments.

Abscisic acid and DCCD were added to the pH 4.5 and pH 5.5 solutions during periods of peak NPPase activity. Concentrations used were the same as for the stomatal aperture experiments (10 uM ABA, 50uM DCCD). Both caused inhibition of NPPase activity; the enhanced NPPase activity at pH 5.5 in epidermal strips taken from plants in the dark was completely abolished by ABA and DCCD (Figure 12).

Addition of KCl to the buffered medium, and deletion of $MgCl_2$, led to no dramatic enhancement of NPPase activity (Figure 13). However, the lower concentrations of K^+ had less NPPase activity than the higher concentrations, and activity seemed to peak near 100 mM KCl, though the results were not conclusive. The concentrations of KCl that stimulated NPPase activity were similar to those that stimulated stomatal opening (Figure 1).

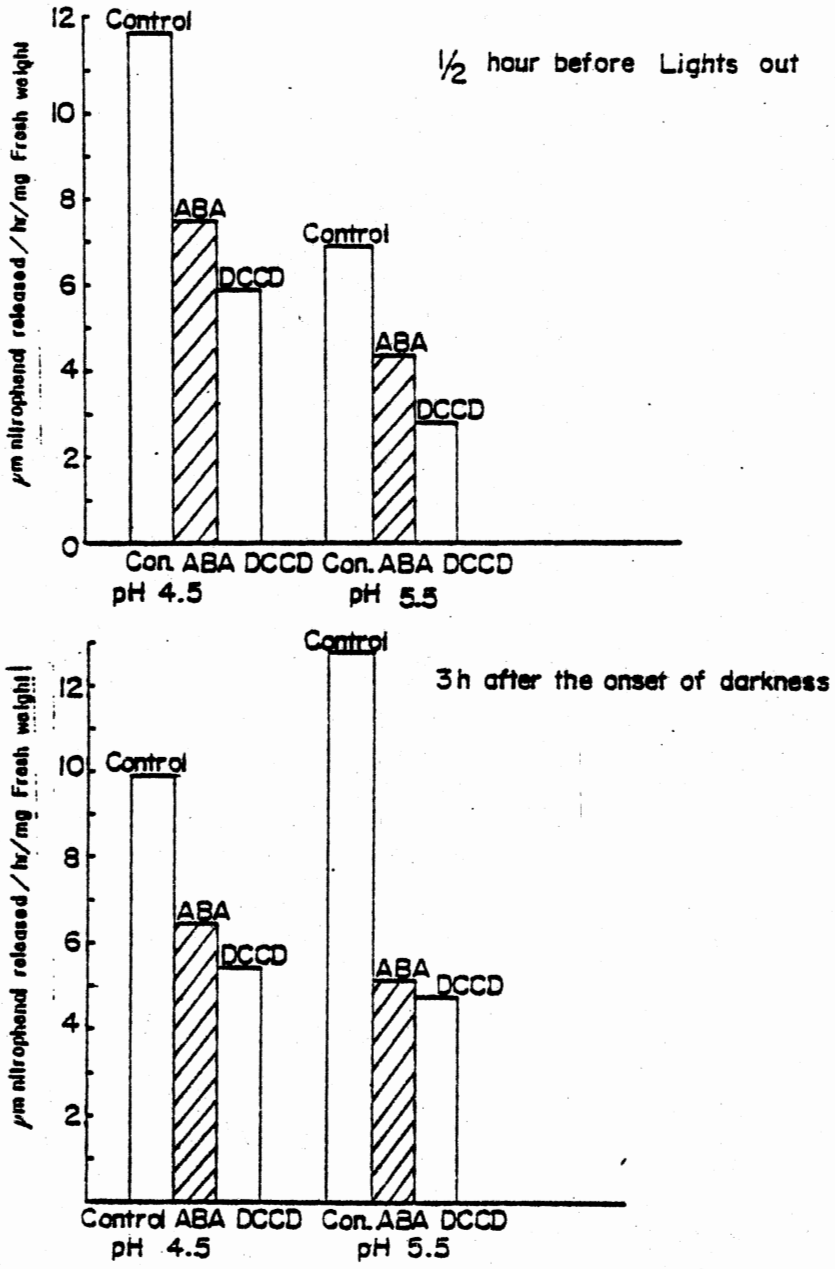

Figure 12. Effect of DCCD and ABA on NPPase Activity. The top graph represents strips taken from plants thirty minutes before the onset of darkness. The lower graph represents strips taken from plants after 3 hr of darkness.

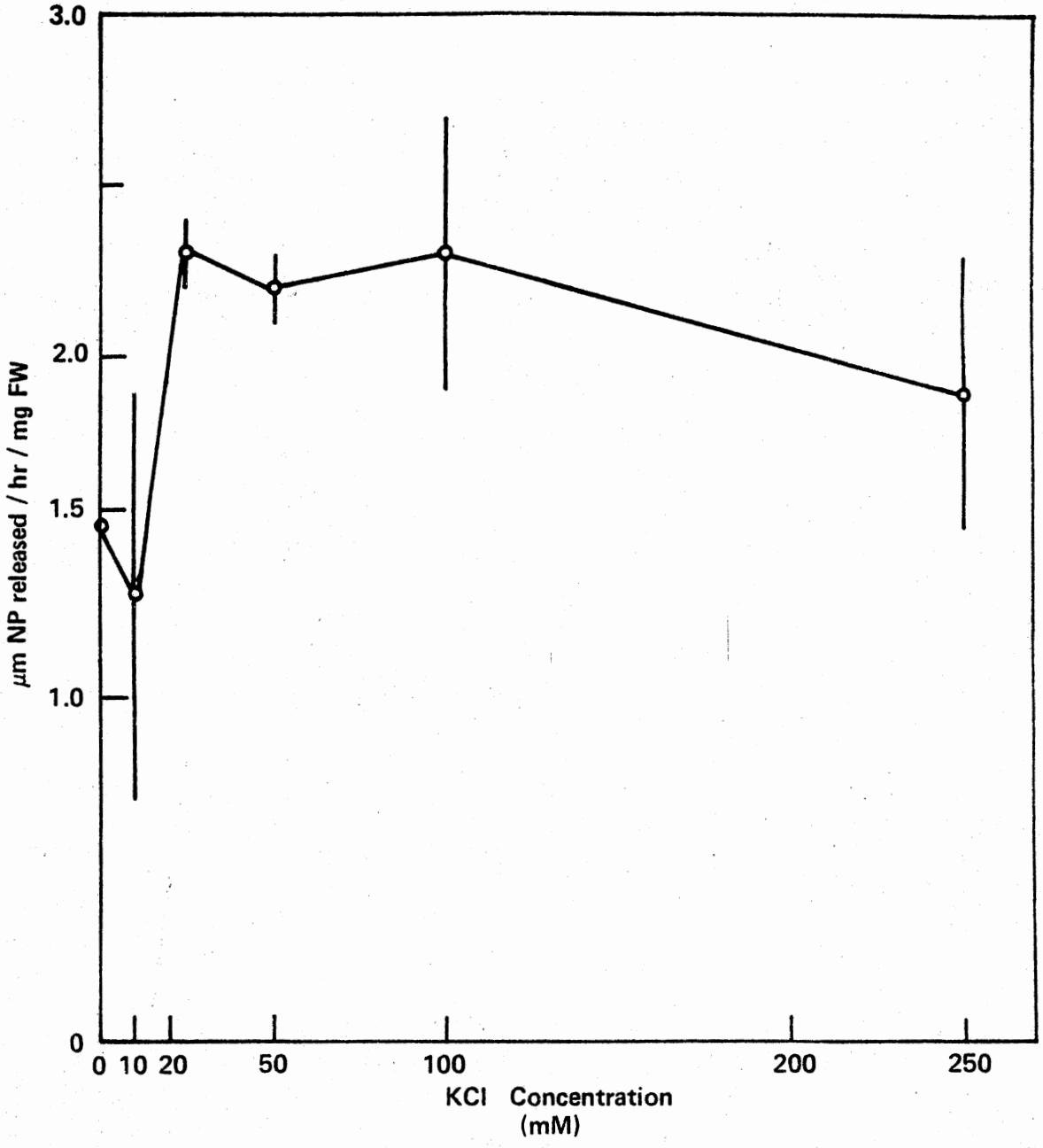

Figure 13. Effects of K^+ on NPPase Activity at pH 7.5. The vertical lines represent the standard deviations of three experiments.

CHAPTER IV

DISCUSSION

The stomata of Crassula argentea appear in many respects to be similar to those of C-3 and C-4 plants. Their opening is associated with an influx of potassium, and it is enhanced by reduced CO_2 levels. On the other hand, the stomata of C. argentea, like other CAM plants, open at night. Early work with epidermal strips of C-3 plants showed neutral or basic pH was conducive to stomatal opening (Small, Clarke, and Crosbie-Baird, 1942). The stomata of C. argentea, on the other hand, respond to low pH by opening, and to neutral or high pH by closing.

The response to low pH could be due to injury of the cells. However, the results of K^+ specific stain show that it is associated with an influx of K^+ to the guard cells. The inhibition by ABA of opening and of the uptake of potassium by guard cells from adjacent subsidiary shows that the response is the same as in uninjured cells.

ABA has been implicated to work in two ways, one to block cyclic photophosphorylation of guard cells chloroplasts (and hence ATP production) and the other to block organic acid formation in guard cells (Raghavendra, Rao and Das, 1976). ABA seems to prevent C. argentea guard cells from actively taking up K^+. This may be accomplished by not only denying the energy needed in the form of ATP, but by disallowing formation of sufficient negative countercharges within the guard

cell. The latter may be important since neither K^+ counter ion used in this study, SO_4^{-2} and CL^{-1}, were important in inducing the opening response.

DCCD is a specific inhibitor of cation-requiring membrane ATPases in bacteria (Abrams and Baron, 1970) and higher plants (Leonard and Hodges, 1973). It has also been shown to inhibit mitochondrial oxidative phosphorylation (Beechey et al., 1967). In the work described here, the apparent localization of potassium in the intercellular spaces of DCCD-treated epidermal strips indicates a different mode of action from ABA. DCCD may act to generally inhibit membrane-bound ATPases involved in K^+ fluxes into and out of guard cells and subsidiary cells, causing K^+ to leak into the intercellular spaces down an electrochemical gradient.

The possibility of potassium uptake by guard cells has only recently gained acceptance. The work of Thomas (1970) with ouabain seemed to implicate involvement of an ATPase. Ouabain is a known inhibitor of animal ATPases though its effects vary from cell type to cell type (Bonting, Caravaggio and Hawkins, 1962). Thomas (1970) showed that ouabain-treated epidermal strips of tobacco closed their stomata, and that exogenous ATP reversed this response. However, Raghavendra et al. (1976) found ouabain to have little or no effect on either ATPase or stomatal movement in Commelina benghalensis. They did, however, extract two ATPases from the epidermis of C. benghalensis and one with a pH optimum of 7.5 seemed to be associated with stomatal opening (Raghavendra, Rao, and Das, 1976). They also found that inhibitors of stomatal opening inhibited the pH 7.5 ATPase isozyme.

Finally, they mentioned preliminary work with histochemical stains showing that the greatest ATPase activity was localized in guard cells.

Fischer (1968) observed that his estimates of K^+ uptake "constitute quantitative evidence in support" of active uptake of K^+ as proposed by Fujino (1967). Fujino observed, like Raghavendra and coworkers, that most epidermal ATPase activity was associated with guard cells and that stomatal opening was a response to K^+ uptake. Raschke (1975), on the other hand, envisages an H^+ pump, with K^+ ions being taken up in exchange for H^+ ions. He believes that ABA inhibits opening by blocking the active excretion of H^+ from guard cells. Fusicoccin, a fungal metabolite which has been shown to stimulate H^+ excretion, enhances stomatal opening and overcomes ABA inhibition of stomatal opening (Raschke, 1975). Raschke (1975) has stated that H^+ expulsion is a primary if not the primary event in the stomatal opening response. He argues that cations follow the electrochemical gradient, and thus K^+ is passively taken up by the guard cells. However, the observations of this study of the response of Crassula argentea stomata to DCCD seem to support the hypothesis that K^+ is actively taken up.

Attempts to repeat the work of Raghavendra, Rao and Das (1976) and isolate epidermal strip ATPase from jade epidermis failed when ATP was used as a substrate, so NPP was used as an alternative method to characterize the high energy phosphate bond-cleaving enzymes of jade plant. NPP is a pseudosubstrate for ATPases and acid phosphatases (Tominaga, 1978; Gache, Rossi and Lazdunski, 1977). In the presence of these enzymes, phosphate is cleaved from the molecule, forming nitrophenol, which is yellow in color at basic pH. The NPPase activity of the jade epidermis was characterized by a low pH optimum at night,

a K_m comparable to that found in animal systems (Albers and Koval, 1972) and inhibition by both ABA and DCCD. Since ABA may inhibit cyclic phosphorylation, and DCCD inhibits membrane-bound ATPases, the NPPases in the epidermal strips of C. argentea may be predominantly ATPases. Another interesting feature of ABA and DCCD inhibition of NPPase activity is that they also inhibit stomatal opening. These observations indirectly suggest that NPPases are actively involved in the control of stomatal movement in Crassula.

The low pH optimum of jade epidermal NPPases in the dark also seems to correlate with the acid-induced opening of stomata. The similarity between the graphs of stomatal aperture as a function of pH and NPPase activity as a function of pH is striking. Again this seems to implicate NPPase involvement in the acid-induced opening, and thus, indirectly, ATPase participation. The role of endogenous acidity in jade leaves may be to maintain stomatal opening. However, the observation of a transient increase in leaf acidity at the onset of darkness suggests that acidity may aid in the initial opening response as well. The correlation with NPPase activity indicates that low pH may act to stimulate the opening response, and then enhance and maintain that response by stimulating NPPase activity.

The addition of K^+ to the NPPase medium did not stimulate NPPase activity above that found when using Mg^{+2}. However, the concentrations of K^+ ($\geq$25 mM) which stimulated stomatal opening in the light also stimulated NPPase activity above the lower concentrations (0 and 10 mM) which did not stimulate opening in the light. This adds credence to the supposition that K^+ uptake is active and is regulated by guard cell membrane-bound ATPases with a low pH optimum at night. The fact

that K^+ did not stimulate NPPase activity above that of Mg^{++} may be due to the possibility that there is no NPPase involvement in stomatal movement, or that most of the NPPase activity represents acid phosphatases which are not involved in K^+ uptake, or, finally, that the experimental conditions may have been unfavorable for observing the response.

In conclusion, it appears that K^+ uptake of Crassula argentea guard cells is stimulated by low pH and that this response may be physiological, it may be active, and it may be regulated by a membrane-bound guard cell ATPase with an acidic pH optimum at night.

The inhibition of opening of acid-treated stomata by ABA indicates a physiological process. K^+-specific staining shows the acid-induced opening to be associated with K^+ influx to the guard cells. This influx is abolished by ABA, with the K^+ being confined to the subsidiary cells in ABA-treated strips. DCCD inhibition of acid treated strips suggests the involvement of a membrane-bound ATPase; K^+ specific stain shows accumulation of K^+ in the intercellular spaces of DCCD treated strips, which suggests that DCCD causes a general inhibition of subsidiary cell and guard cell ATPases. Characterization of epidermal ATPases and acid phosphateses, using the pseudosubstrate NPP, shows a low pH optimum at night, low activity during the day, and inhibition by ABA and DCCD. Basic pH also inhibits the activity of the NPPases.

LITERATURE CITED

Abrams, A. and C. Baron. 1970. Inhibitory action of carbodiimide on bacterial membrane ATPase. Biochem. Biophys. Res. Commun. 41: 853-863.

Albers, R. W. and George J. Koval. 1972. Sodium-Potassium-activated Adenosine Triphosphatase. VII. Concurrent inhibition of Na^+-K^+-Nitrophenylphosphatase activities. J. of Biol. Chem. 247:3088-3092.

Allaway, W. G. 1973. Accumulation of malate in guard cells of Vicia faba during stomatal opening. Planta 110:63-70.

Aylor, D. E., J. P. Parlange and A. D. Kirkorian. 1973. Stomatal mechanics. Am. J. Bot. 60:163-171.

Beechey, R. B., A. M. Robertson, C. I. Holloway, and I. G. Knight. 1967. The properties of dicyclohexylcarbodiimide as an inhibitor of oxidative phosphorylation. Biochemistry 6:3867-3879.

Black, C. C. and S. Williams. 1976. Plants exhibiting characteristics common to CAM. In R. H. Burris and C. C. Black, eds., CO_2 Metabolism and Plant Productivity. University Park Press, Baltimore, MD. pp. 235-250.

Bonting, S. L., L. L. Caravaggio, and N. M. Hawkins. 1967. Studies in sodium-postassium-activated adenosinetriposphatase. IV. Correlation with cation transport sensitive to cardiac glycosides. Archs. Biochem. Biophys. 98:413-419.

Bowling, D. J. F. 1976. Malate switch hypothesis to explain the action of stomata. Nature 262:393-394.

Crews, C. E., S. L. Williams, H. M. Viens, and C. C. Black. 1976. Changes in the metabolism and physiology of CAM plants grown in controlled environments. In R. H. Burris and C. C. Black, eds., CO_2 Metabolism and Plant Productivity. University Park Press, Baltimore, MD. pp. 235-250.

Dayanandan, P. and P. B. Kaufman. 1975. Stomatal movements associated with potassium fluxes. Amer. J. Bot. 62:221-231.

DeMichele, D. W. and P. J. H. Sharpe. 1973. An analysis of the mechanics of guard cell motion. J. Theor. Biol. 41:77-96.

Esau, K. 1965. Plant Anatomy. Wiley and Sons, New York. p. 158.

Fischer, R. A. 1968. Stomatal opening in isolated epidermal strips of Vicia faba. I. Response to light and to CO_2-free air. Plant Physiol. 43:1947-1952.

Fischer, R. A. and T. C. Hsiao. 1968. Stomatal opening in isolated epidermal strips of Vicia faba. II. Responses to KCl concentration and the role of potassium absorption. Plant Physiol. 43: 1953-1958.

Fujino, M. 1967. Role of adenosinetriphosphate and adenosinetriphosphatase in stomatal opening. Sci. Bull. Fac. Educ. Nagasaki Univ. 18:1-47.

Gache, C., B. Rossi, and M. Lazdunski. 1977. Mechanistic analysis of the (Na^+, K^+) ATPase using new pseudosubstrates. Biochem. 16: 2957-2965.

Goh, C. J., P. N. Avadhani, C. S. Loh, C. Hanegraaf and J. Anditti. 1977. Diurnal stomatal and acidity rhythms in orchid leaves. New Phytol. 78:365-372.

Hartsock, T. L. and P. S. Nobel. 1976. Watering converts a CAM plant to daytime CO_2 uptake. Nature 262:574-576.

Hiron, R. W. P. and S. T. C. Wright. 1973. The role of endogenous abscisic acid in the response of plants to stress. J. of Exper. Botany 24:769-781.

Hodges, T. K., R. T. Leonard, C. E. Bracker, and T. W. Keenan. 1972. Purification of an ion stimulated adenosine triphosphatase from plant roots: association with plasma membranes. Proc. Nat. Acad. Sci. USA 69:3307-3311.

_____. 1976. ATPases associated with membranes of plant cells. In A. Pirson and M. H. Zimmermann, eds., Encyclopedia of Plant Physiology (New Series) Vol. IIa. Springer-Verlag, New York. pp. 260-283.

Hsiao, Theodore C. 1973. Plant responses to water stress. In W. R. Briggs, P. B. Green, and R. C. Jones, eds., Annual Review of Plant Physiology, Vol. 24. pp. 519-570.

Immamura, S. 1943. Untersuchungen Über den Mechanismus der Turgorschwankung der Spaltöffnungsschliesszellen. Japan. J. Botany 12:251-347.

Kluge, M. 1976. Models of CAM regulation. In R. H. Burris and C. C. Black, CO_2 Metabolism and Plant Productivity. University Park Press, Baltimore, MD. pp. 205-216.

Kriedemann, P. E., B.R. Loveys, L. Fuller, and A. C. Leopold. 1972. Abscisic acid and stomatal regulation. Plant Physiol. 49:842-847.

Lai, Y. F. and J. E. Thompson. 1971. The preparation and properties of an isolated plant membrane fraction enriched in (Na^+-K^+)-stimulated ATPase. Biochem. Biophys. Act. 233:84-90.

Lang, L. ed. 1962. Absorption spectra in the ultraviolet and visible region, Vol. III. Wiley and Sons, New York. pp. 96-97.

Larcher, W. 1973. Physiological Plant Ecology. Springer-Verlag, Berlin. pp. 30-33.

Leonard, R. T. and T. K. Hodges. 1973. Characterization of plasma membrane-associated adenosine triphosphatase activity of oat roots. Plant Physiol. 52:6-12.

Loveys, B. R. and P. E. Kriedemann. 1973. Rapid changes in abscisic acid-like inhibitors following alterations in vine leaf water potential. Physiol. Plant. 28:476-479.

Macallum, A. B. 1905. On the distribution of potassium in animal and vegetable cell. J. Physiol. 32:95-128.

Majernik, Ondrej and T. A. Mansfield. 1972. Stomatal response to raised atmospheric CO_2 concentrations during exposure of plants to SO_2 pollution. Environ. Pollution 3:1-7.

McWilliams, E. T. 1970. Comparative rates of dark CO_2 uptake and acidification in the Bromeliacae, Orchidaceae, and Euphorbiaceae. Bot Gaz. 131:285-290.

Most, B. H. 1971. Abscisic acid in immature apical tissue of sugar cane and in leaves of plants subjected to drought. Planta 101: 67-75.

Nishida, K. 1963. Studies on stomatal movement of Crassulacean plants in relation to the acid metabolism. Physiol. Plant. 16:254-298.

Noggle, G. R. and G. J. Fritz. 1976. Introductory Plant Physiology. Prentice-Hall, Inc., New York. pp. 87-190.

Osmond, C. B. 1978. Crassulacean acid metabolism: a curiosity in context. In W. R. Briggs, P. B. Green, and R. L. Jones, eds. Annual Review of Plant Physiology, Vol. 29. Wiley and Sons, New York. 379-414.

Pallaghay, C. K. and K. Rashke. 1972. No stomatal response to ethylene. Plant Physiol. 49:275-276.

Pucher, G. W., C. S. Leavenworth, W. D. Ginter, and H. B. Vickery. 1947. Studies in the metabolism of Crassulacean plants: the duirnal variation in organic acid and starch content of Bryophyllum calycinium. Plant Physiol. 22:360-375.

Raghavendra, A. S., I. M. Rao, and V. S. R. Das. 1976. Characterization of abscisic acid inhibition of stomatal opening in isolated epidermal strips. Plant Sci. Letters 6:111-115.

_____. 1976. Adenosine triphosphatase in epidermal tissue of Commelina benghalensis: possible involvement of isozymes in stomatal movement. Plant Sci. Letters 7:391-396.

Raschke, K. 1970. Temperature dependence of CO_2 assimilation and stomatal aperture in leaf sections of Zea mays. Planta 91:336-363.

_____. 1972. Saturation kinetics of the velocity of stomatal closing in response to CO_2. Plant Physiol. 49:229-234.

_____. 1975. Stomatal Action. In W. R. Briggs, P. B. Green, and R. C. Jones. Annual Review of Plant Physiology, Vol. 26. Wiley and Sons, New York. pp. 309-340.

Scarth, G. W. 1932. Mechanism of the action of light and other factors on stomatal movement. Plant Physiol. 7:481-504.

Schnable, H. and H. Ziegler. 1977. The mechanism of stomatal movement in Allium apa L. Planta 136:37-43.

Small, J., M. I. Clarke, and J. Crosbie-Baird. 1942. pH phenomena in relationship to stomatal opening. II-V Proc. Royal Soc. Edinburgh. Ser. B 61:233-266.

Stälfelt, M. G. 1929. Die Abhängigkeit des Spaltöfnungsreaktionen von der Wasserbilanz. Planta 8:287-340.

Szarek, S. R. and J. H. Troughton. 1976. Carbon isotope ratios in Crassulacean acid metabolism plants. Plant Physiol. 58:367-370.

Thomas, D. A. 1970. The regulation of stomatal aperture in tobacco leaf epidermal strips. II. The effect of ouabain. Australian J. Biol. Sci. 23:981-989.

_____. 1971. The regulation of stomatal aperture in tobacco leaf epidermal strips. III. The effect of ATP. Australian J. Biol. Sci. 24:689-707.

Tominaga, N. 1978. Comparative studies on the effects of anions on ATPase and acid phosphatase activities from different sources. Plant and Cell Physiol. 19:627-636.

Willmer, C. M. and J. E. Pallas. 1973. A survey of stomatal movements and associated potassium fluxes in the plant kingdom. Can. J. Bot. 51:37-32.

VITA

Mark Vernon Loucks

Candidate for the Degree of

Master of Science

Thesis: STUDIES ON FACTORS CONTROLLING THE OPENING OF STOMATA IN THE CAM PLANT CRASSULA ARGENTEA

Major Field: Botany

Biographical:

Personal Data: Born in Ft. Belvoir, Virginia, November 16, 1953, the son of Mr. and Mrs. Keith W. Loucks.

Education: Graduated from Ponca City High School, Ponca City, Oklahoma, in 1971; received the Bachelor of Science degree in Biological Sciences from Oklahoma State University, Stillwater, Oklahoma, in 1976; completed requirements for the Master of Science degree at Oklahoma State University in May, 1979.

Professional Experience: Orderly at St. Joseph's Hospital, Ponca City, Oklahoma, part-time from 1971 to 1974; graduate teaching assistant and research assistant in the School of Biological Sciences, 1976-1979.

Professional Organizations: American Society of Plant Physiologists, the Society of the Sigma Xi.