

# Electrochemical Aging Responses in *Pisum*: Cellular Adaptations or Recovery from Injury?<sup>1</sup>

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## ABSTRACT

Following excision, etiolated epicotyl segments of *Pisum sativum* L. cv. Alaska exhibit a marked hyperpolarization of membrane potential which is followed by a linear accumulation of K<sup>+</sup> when segments are incubated in Higinbotham nutrient solution. Segments aged for several hours and then reexcised display only a slight depolarization of membrane potential and no delay in ion accumulation; thus, recovery from injury appears an unlikely explanation for these responses. Substances originating in either the plumule or the cotyledons do not seem to be directly involved in these "aging" responses. However, locally produced substances, such as ethylene, or substances originating in the roots have not been eliminated as causative factors. Cold temperatures and cycloheximide prolong the lag in K<sup>+</sup> accumulation indicating a metabolic explanation for the induced K<sup>+</sup> accumulation. However, similar specific activities of plasma membrane-bound ATPase were found in isolates from fresh and aged epicotyl segments. Reactivation of an ion transport mechanism, perhaps responsible for the osmotic control of growth in immature cells, is suggested as a possible explanation for the pattern of ion accumulation characteristic of excised pea epicotyl tissue.

The pea plant, specifically its epicotyl, is used in a variety of biochemical and biophysical investigations. Many studies involve excised tissue; the question frequently arises as to whether the results can be extrapolated to conditions in the intact plant. This question has particular relevance for studies with peas since a number of apparent adaptive changes (often labeled "aging"<sup>2</sup> responses) have been observed when segments of this plant are employed (7, 14, 25).

Macklon and Higinbotham (14) were the first to report that freshly excised segments of *Pisum* epicotyl exhibit a marked hyperpolarization of membrane potential and a delayed accumulation of ions during the initial hours of incubation in Higinbotham nutrient solution. Higinbotham and Pierce (7) subsequently showed that the rate of ion accumulation following this lag period was quite linear and that K<sup>+</sup> accumulation was de-

pendent upon concomitant anion accumulation. Hendrix and Higinbotham (4) demonstrated that the ion accumulation process in this tissue is sensitive to various sulfhydryl-directed inhibitors.

Previous studies indicate that a transport system in *Pisum* epicotyl cells is either being activated or created following excision. Proper interpretation of these transport studies with segments depends upon a determination of whether time-dependent changes represent actual adaptations to a new cellular environment or are indicative of a system returning to normal functioning following injury. The experiments reported here were undertaken to determine which of these possibilities is most tenable with respect to explaining the electrochemical aging responses of *Pisum* epicotyl segments.

## MATERIALS AND METHODS

Seeds of *Pisum sativum* L. cv. Alaska were surface sterilized with 20% Clorox, soaked for 2 to 4 h in distilled H<sub>2</sub>O and planted in trays of vermiculite. The plants were grown in the dark for 7 days at 25 C. The upper 1.5 cm of each epicotyl was then discarded and the next two 1-cm segments were excised from the third internode. Twenty segments (approximately 0.75 g) were used for each sample in ion uptake experiments. Unless otherwise indicated, excised segments were incubated on a reciprocating shaker in 100 ml of a solution referred to as 1× (6). The 1× contained, in mM, KCl, 1.0; NaH<sub>2</sub>PO<sub>4</sub>, 0.90; Na<sub>2</sub>HPO<sub>4</sub>, 0.05; Ca(NO<sub>3</sub>)<sub>2</sub>, 1.0 and MgSO<sub>4</sub>, 0.25. The pH of this solution is approximately 5.4. All uptake and electrical potential measurements were made at 20 C, except where noted.

Membrane-bound ATPase was isolated by density gradient centrifugation using linear sucrose gradients (5, 21). Gradient fractions with densities corresponding to 30.5 to 32.5% sucrose were pooled and used in these assays (5). ATPase activity was assayed at pH 6.0 or 8.2 and 38 C using a reaction mixture consisting of 3 mM MgCl<sub>2</sub>, 3 mM Tris-ATP, 33 mM Tris-Mes, and 50 mM KCl or KNO<sub>3</sub>, where appropriate (5, 21). The Pi released during incubation or membrane fractions with ATP was measured by the technique of Fiske and SubbaRow (1).

Potassium analyses of hot water extracts of the tissue were performed by flame-emission spectrophotometry. For these analyses, three or four replicates of tissue samples were used.

Ethylene and ethane emission from epicotyl segments was determined by sampling the headspace above tissue segments in 25-ml Erlenmeyer flasks sealed with serum stoppers. Segment incubation medium consisted of 1× and 150 μM chloramphenicol. To test the effect of O<sub>2</sub> tension upon the evolution of these gases, the headspace O<sub>2</sub> tension in several flasks was increased by substitution of 2 ml of pure O<sub>2</sub> for an equal amount of air, at the time of sealing. Ethylene and ethane in the headspace were determined by removal of 0.2-ml samples which were injected

<sup>1</sup> This paper is dedicated to the memory of Dr. Noe Higinbotham, in whose laboratory both authors first experienced the rewards and frustrations of ion transport research.

<sup>2</sup> Abbreviations and definitions: Aging, we use this term to refer to physiological changes that occur as the result of tissue excision as opposed to changes associated with senescence; 1×, Higinbotham nutrient solution at normal concentration; PD, potential difference (transmembrane electrical potential).

into a Carle model 311 gas chromatograph. The chromatograph was equipped with a Porapak T column operated at 70 C; a flame ionization detector was employed to quantitate these gases.

**RESULTS**

**Effect of Increasing pH on K<sup>+</sup> Accumulation and Cell Potentials.** Figure 1 illustrates the changes in cell electrical potential and the pattern of ion accumulation characteristic of pea epicotyl tissue following excision. These data were obtained using 1X to which 1 mM NaHCO<sub>3</sub> had been added to raise the pH from 5.4 to 6.8. Poole (23) has reported a more rapid hyperpolarization of cell potential occurs in freshly excised beet root tissue when the pH of the solution is elevated with bicarbonate. The pattern of hyperpolarization shown in Figure 1, however, was comparable to control values in 1X (data not shown) and was similar to data reported previously for 1X (7). The rate of net K<sup>+</sup> uptake following the lag period was slightly accelerated when bicarbonate was present, compared to controls in 1X (data not shown), but the length of the lag period was unaffected.

**Effect of More Extensive Tissue Damage on Pattern of K<sup>+</sup> Accumulation.** If the aging responses shown in Figure 1 are the result of recovery from injury, increasing the ratio of damaged to nondamaged tissue could lengthen the recovery period. Figure 2 summarizes the results of an experiment in which epicotyl segments were cut longitudinally, as well as transversely. The lag in

K<sup>+</sup> accumulation was not extended by the additional tissue damage and the rate of K<sup>+</sup> accumulation following the lag period was actually enhanced. Longitudinal sectioning presumably increases rates of diffusion of K<sup>+</sup> to uptake sites.

**Effects of Reinjury on K<sup>+</sup> Accumulation.** Table I summarizes the effects of a second excision on K<sup>+</sup> accumulation. In this experiment some segments were sliced longitudinally after 8 h of aging in 1X. This resulted in a slight reduction in K<sup>+</sup> accumulation compared to that of sections sliced longitudinally at 0 h (Table I). If the lag in K<sup>+</sup> accumulation (Fig. 1) is due to recovery from injury, reexcision after 8 h should have delayed K<sup>+</sup> accumulation for another several hours and thus have resulted in a K<sup>+</sup> content of less than 30 μmol/g (estimated by shifting the upper line in Fig. 2 to the right 8 h). Since accumulation was only slightly affected (Table I), the lag in K<sup>+</sup> accumulation can not be due simply to recovery from injury.

**Effect of Reinjury on Cell PD.** Table II summarizes PD data collected from epicotyl segments which had been aged for about 23 h in 1X and then had a 0.2-cm section excised from each end. A slight depolarization of cell potential occurred (from a control value of -135 to -117 mv) as the result of the second excision. This degree of depolarization is insufficient to account for the dramatic hyperpolarization of potential exhibited in Figure 1, since freshly excised segments have cell potentials of about -90 mv after 2 h in 1X (7). Therefore, recovery from injury appears to be an inadequate explanation for either of the aging responses shown in Figure 1.

**Influence of Plumules and Cotyledons on K<sup>+</sup> Accumulation.** Isolating segments from the normal translocation stream may eliminate the source of some hormone that is suppressing an existing cellular transport system in intact epicotyls. Figure 3 summarizes the results of an experiment in which the plumules were not detached from the subterminal segments used in uptake studies until after the designated period of ion accumulation. The presence of the plumule had no detectable effect on the pattern of K<sup>+</sup> accumulation, indicating that enhanced K<sup>+</sup> accumulation

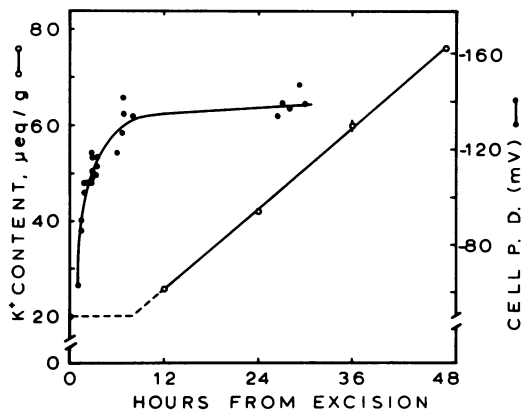


FIG. 1. Changes in cell potential difference and K<sup>+</sup> content of pea epicotyls after excision. Segments were incubated in 1X, adjusted to pH 6.8 with 1 mM NaHCO<sub>3</sub>. Standard deviations are indicated on the K<sup>+</sup> content data, in this and subsequent figures, where the deviations are greater than the symbol size.

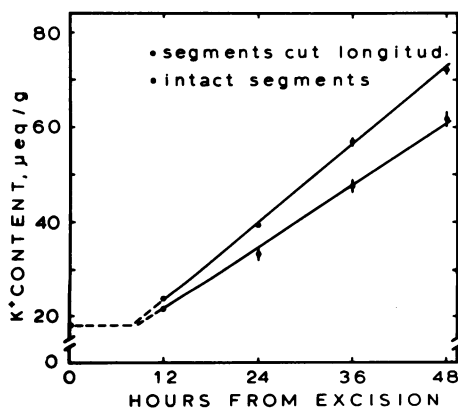


FIG. 2. Effects of longitudinal slicing upon the K<sup>+</sup> content of pea epicotyl segments. Some segments (●) were cut longitudinally at 0 h while others (○) were only cut transversely. All segments were incubated in 1X, with solution changes at 12-h intervals.

Table I. Influence of a Second Excision, after Aging, upon K<sup>+</sup> Accumulation in Etiolated *Pisum* Epicotyl Segments

Segments were aged in 1X, with solution changes at 8 h. The data are averages of four replicates ± SD.

Treatment	Time From Initial Excision		
	0 h	8 h	24 h
	μmol K <sup>+</sup> /g fresh wt		
Transverse cuts at 0 h	25.6 ± 0.2	24.3 ± 0.5	36.4 ± 0.6
Transverse + longitudinal cuts at 0 h	25.6 ± 0.2	25.6 ± 0.9	46.2 ± 1.3
Transverse cuts at 0 h, longitudinal cut at 8 h	25.6 ± 0.2	24.3 ± 0.5	42.0 ± 1.4

Table II. Effect of a Second Excision, After Aging, upon the PD of Etiolated *Pisum* Epicotyl Segments

Segments were aged in 1X with solution changes after 12 h. Second excisions (2-mm sections removed from tips of segments) were made at about 23 h after the initial excision. The PD is the average value obtained from the number of different segments indicated. All values are ± SD.

Treatment	Average Time Since First Excision	Average Time Since Second Excision	Average PD	Number of Segments
	h			
One excision	25 ± 2		135 ± 6	31
Two excisions	25 ± 2	2 ± 1	117 ± 13	29

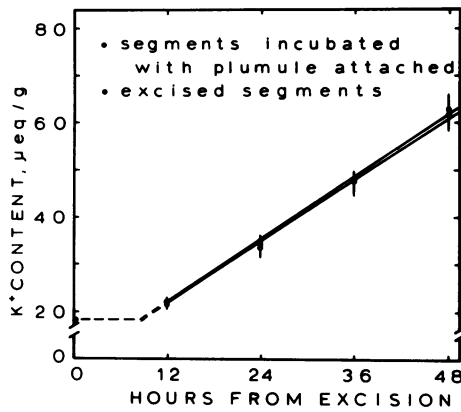


FIG. 3. Effect of leaving the plumule attached upon the pattern of ion accumulation in subterminal pea epicotyl segments. Segments labeled as "excised" were cut into 1-cm segments at 0 h. Segments with plumule attached consisted of the terminal 3.5 cm of the epicotyl. The terminal 1.5 cm of the plumular segments was discarded after the designated incubation period. All segments were incubated in 1X, with solution changes at 12-h intervals.

Table III. Emission of Ethylene and Ethane by Etiolated *Pisum* Epicotyl Segments and Plumules

Segments were excised into 25-ml Erlenmeyer flasks containing 5 ml 1X, plus 150  $\mu$ M chloramphenicol and air or O<sub>2</sub>-supplemented air as indicated. The flasks were shaken on a rotary shaker at 23 C for 18 h and the headspace above the tissue was sampled for ethylene and ethane by withdrawal with syringe. All tissue weights were 3.0 g; the treatment, labeled plumules + stems, contained 1.5 g of each tissue. Oxygenated flasks had 2 ml of O<sub>2</sub> injected at the time of sealing, following the removal of an equal volume of air from the headspace. Data represent the averages of two replicates.

Treatment	Ethylene	Ethane
	<i>nl/g fresh wt</i>	
Epicotyl segments + air	2.0	8.7
Epicotyl segments + O <sub>2</sub>	14.2	2.0
Epicotyl + cycloheximide + air	1.5	9.7
Epicotyl + cycloheximide + O <sub>2</sub>	7.2	3.1
Plumules + air	3.1	24.6
Plumules + epicotyls + air	2.0	11.3

occurs in the subterminal segments whether or not the plumule is attached.

Removal of cotyledons from seedlings 12 h prior to excision of the epicotyl segments had no effect on the pattern of K<sup>+</sup> uptake by the epicotyl segments. No satisfactory method was devised for testing possible root influences.

**Effect of Excision and Aging on Endogenous Ethylene Production.** It has been suggested (24) that a volatile entity may account for lag phases similar to the lag in K<sup>+</sup> accumulation noted here. Further, changes in ethylene production in pea tissues are known to occur in response to stress (10), wounding (25), or application of auxin (26). Preliminary experiments (Table III) failed to show any correlation between ethylene or ethane evolution from epicotyl segments (during aging in 1X for 18 h) and the pattern of delayed ion accumulation. Further, addition of cycloheximide at concentrations known to inhibit K<sup>+</sup> accumulation (3) did not alter the pattern of ethylene production.

An increase in ethylene but a decrease in ethane production was noted when 2 ml of O<sub>2</sub> was substituted for an equivalent amount of air in stoppered flasks containing epicotyl segments (Table III), indicating that aeration of nutrient solution could be an important factor in regulating emission of these gases by this tissue.

**Effect of Cold Temperature on Pattern of K<sup>+</sup> Accumulation.**

Figure 4 indicates that incubation of segments at 4 C extends the lag in K<sup>+</sup> uptake by a time equivalent to the period of cold exposure. The rate of K<sup>+</sup> accumulation following cold exposure is not affected by this treatment.

**Effect of Cycloheximide, Benzyladenine, and Chloramphenicol on K<sup>+</sup> Uptake.** A bactericidal concentration of chloramphenicol had no effect on K<sup>+</sup> accumulation (Fig. 5). Benzyladenine did not affect the length of the lag period but did reduce subsequent K<sup>+</sup> accumulation. A concentration of cycloheximide, known to inhibit protein and nucleic acid synthesis (16), prevented K<sup>+</sup> accumulation but permitted the cells to retain their original K<sup>+</sup> levels. This concentration of cycloheximide has been shown to have no effect on cell PDs in aged pea epicotyl tissue (3).

**Effect of Aging on Membrane-bound ATPase.** Experiments with cold temperature (Fig. 4) and cycloheximide (Fig. 5) suggest the

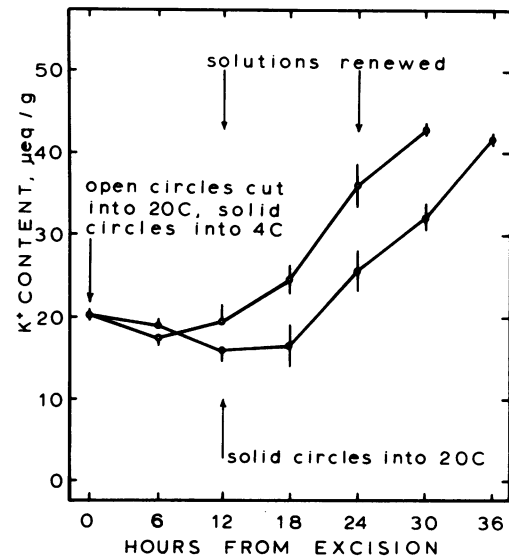


FIG. 4. Effect of aging at 4 C upon the pattern of K<sup>+</sup> accumulation in pea epicotyl segments. Segments incubated at 4 C (●) were transferred to 20 C after 12 h. All segments were incubated in 1X, with solution changes at 12-h intervals.

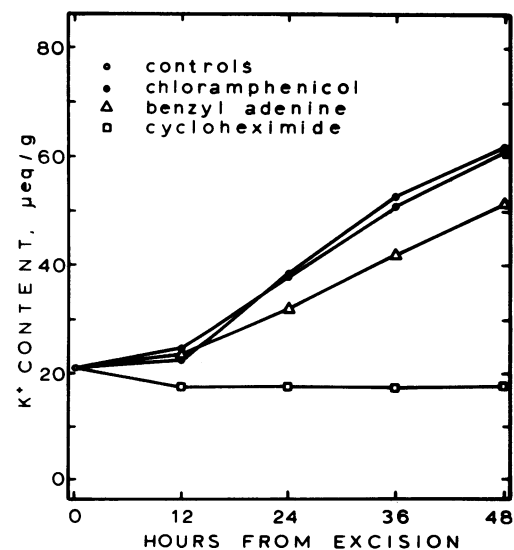


FIG. 5. Effect of chloramphenicol, benzyladenine, and cycloheximide upon K<sup>+</sup> accumulation in pea epicotyl segments. All segments were incubated in 1X, with solution changes at 12-h intervals; where indicated, 150  $\mu$ M chloramphenicol, 5  $\mu$ M benzyladenine, or 8.6  $\mu$ M cycloheximide was added to the 1X at 0 h.

possibility that a transport system is created during the normal 8-h lag in  $K^+$  uptake. Membrane-bound ATPases have been proposed as possible transmembrane ion conveyors (8). For instance, Leonard and Hanson (13) found a positive correlation between aging and levels of a plasma membrane ATPase in corn roots. The distribution of membrane-bound ATPase from freshly excised epicotyl segments on a 20 to 45% linear sucrose gradient has been reported elsewhere (21). Similar ATPase distribution patterns to those shown in the previous reference have been obtained from epicotyl segments aged for 24 h in  $1\times$  (Pierce and Hendrix, unpublished). Specific activities of plasma membrane-bound ATPase (21) from fresh and aged epicotyl segments are also similar (Table IV). The addition of  $10\ \mu\text{M}$  cycloheximide to the incubation medium 12 h prior to membrane isolation generally depressed ATPase specific activity except when  $\text{KNO}_3$  was used as the stimulating salt.

## DISCUSSION

Aging responses involving ion transport have been observed with tissues excised from a variety of plants. In most instances, enhanced ion uptake is either preceded by or accompanied by a hyperpolarization of membrane potential. Tissue excision seems to be the signal which initiates these changes. At least three distinguishable types of aging responses have been recorded: (a) an enhancement of ion uptake requiring up to 24 h for full development, usually accompanied by an increased respiratory rate, which is characteristic of certain storage tissues (27); (b) an enhancement of ion uptake commencing immediately upon excision, accompanied by a decrease or slight increase in respiration rate, which has been reported in barley (9, 17) and corn roots (12); and (c) an enhancement of ion accumulation commencing after a predictable several-hour lag, not accompanied by respiratory alterations, such as that reported for pea stem (7, 18). The three aging types mentioned above may represent alternative solutions to the same problem: bringing a system into electrochemical steady state with a new environment.

Not all excised plant tissues show such aging responses. Unlike pea epicotyl segments, segments from pea roots, taken from plants that have been grown hydroponically for 5 days in  $1\times$ , do not exhibit either a hyperpolarization of cell potential or an enhancement of ion uptake (20).

Just what causes aging responses in those excised tissues which exhibit them? Macklon and Higinbotham (15) demonstrated, through comparison of efflux rates in fresh and aged epicotyl tissue, that the lag in  $K^+$  accumulation (Fig. 1) is not attributable

Table IV. Effect of Aging, in the Presence and Absence of Cycloheximide, upon the Response of *Pisum* Plasma Membrane ATPase to  $\text{KCl}$  and  $\text{KNO}_3$

Pooled fractions, from the density gradient position (30.5–32.5% sucrose) identified as corresponding to the location of *Pisum* plasma membrane (21), were used in these ATPase assays. The ATPase reaction mixtures contained 3 mM  $\text{MgCl}_2$ , 3 mM ATP, 33 mM Tris-Mes at the pH noted and, where indicated, 50 mM  $\text{KCl}$  or  $\text{KNO}_3$ . Prior to membrane isolation, tissue was aged for 12 h in aerated  $1\times$  or  $1\times + 10\ \mu\text{M}$  cycloheximide. Data are the means  $\pm$  SD of five replicates.

Assay Conditions	pH	ATPase		
		Fresh	Aged	Cycloheximide
$\mu\text{mol/mg protein}\cdot\text{h}$				
– K	6.0	32.4 $\pm$ 1.2	33.6 $\pm$ 1.1	24.9 $\pm$ 3.0
– K	8.2	33.5 $\pm$ 2.5	29.6 $\pm$ 2.1	20.5 $\pm$ 0.4
+ KCl	6.0	46.5 $\pm$ 3.9	40.5 $\pm$ 1.8	34.7 $\pm$ 3.2
+ KCl	8.2	30.9 $\pm$ 1.6	30.1 $\pm$ 2.2	25.1 $\pm$ 0.6
+ $\text{KNO}_3$	6.0	37.5 $\pm$ 2.9	33.6 $\pm$ 1.8	31.9 $\pm$ 1.5
+ $\text{KNO}_3$	8.2	19.3 $\pm$ 1.8	15.8 $\pm$ 0.9	14.8 $\pm$ 2.1

to a higher rate of  $K^+$  leakage during the lag period than during the subsequent period of ion accumulation. They also demonstrated that the hyperpolarization of PD was not due to excision of segments into a concentrated salt solution since similar patterns of hyperpolarization occurred when segments were aged in  $1\times$  or distilled  $\text{H}_2\text{O}$ .  $K^+$  accumulation also occurs (after a predictable lag) whether the tissue is aged in salt solution or water. Kendrick and Hillman (11) found no changes in membrane permeability to  $K^+$  in etiolated pea epicotyl that could be attributed to phytochrome or to deetiolation.

Data reported here (Tables I and II, Fig. 2) indicate that recovery from injury is an inadequate explanation for either the hyperpolarization of cell PD or the delayed onset of  $K^+$  accumulation in pea epicotyl segments. Plugging of xylem during aging (9) is an unlikely explanation for enhanced ion accumulation in this tissue, since reexcision of segments after aging (Table I) has a negligible effect on  $K^+$  accumulation. Isolation of epicotyl segments from the normal flow of substances from the plumule and cotyledons does not appear to explain enhanced  $K^+$  accumulation (Fig. 3). The possibility remains, however, that some chemical originating in the roots has a depressing effect on cell potential and ion accumulation in epicotyl cells until the tissue is excised. In fact, Parrondo and Smith (19) demonstrated that removal of the tip of corn roots resulted in increased  $\text{Rb}^+$  uptake following aging in  $\text{CaSO}_4$ ; this enhancement decreased with increasing distance from the root tip.

Transient changes in endogenous ethylene levels have been noted when epicotyl segments are excised (25). Jaffe (10) has suggested that even a slight mechanical perturbation of etiolated pea stems and bean hypocotyls may cause membrane changes which can induce ethylene synthesis which, in turn, leads to morphogenic changes in these plants. Preliminary experiments (Table III) have thus far not shown any obvious correlation between delayed ion accumulation (Fig. 1) and changes of ethylene production following excision. However, transient changes (25), too short to be detected in these experiments, might act as a trigger for the aging responses considered here.

The extension of the lag in ion accumulation by incubation of pea epicotyl segments at  $4\text{C}$  or in cycloheximide (Figs. 4, 5) indicates that there may be some adaptive metabolic explanation for the delayed induction of ion accumulation. These same data eliminate leaching of an inhibiting substance as the explanation for the delay in ion accumulation, since neither cold nor cycloheximide would be expected to block simple diffusion from extracellular space. These agents might block the synthesis of an enzyme responsible for the *in situ* degradation of an inhibiting substance or they might block the creation of, or the activation of, a membrane transport system. For example, it has been proposed that the "augmented" uptake observed with excised corn roots (12, 13) involves the addition of preformed units (proteins?) to an existing  $K^+$  absorption apparatus (2). This conclusion was based, in part, upon results obtained with cycloheximide and, as the results reported here, meaningful interpretation depends upon the specificity of this inhibitor (16).

Rains (24) has discussed the importance of eliminating bacterial effects in long term ion transport studies. The absence of a chloramphenicol effect (Fig. 5) indicates that bacterial contamination is not the explanation for enhanced ion accumulation in pea epicotyl segments.

Tissue aging did not alter the specific activity of an ATPase previously identified as being associated with a plasma membrane-enriched fraction prepared from this tissue (21). Further, we did not observe significant alterations in total ATPase activity in pea epicotyl tissue due to aging. These results (Table IV) do not preclude the possibility that activation of this ATPase, following excision, leads to a hyperpolarization of cell potential or delayed ion accumulation. The lack of cation specificity exhibited by this

preparation (5), sets it apart from most other higher plant plasma membrane ATPases (8).

Preliminary experiments (Pierce, unpublished) have shown that the plumule segments, normally discarded in our experiments, accumulate ions at a rate which parallels growth. When accumulation of  $K^+$  in these segments is calculated based upon final fresh weight,  $K^+$  content (between 0 and 48 h after excision) remains stable. When accumulation is calculated using initial fresh weights (weights at time of excision) an apparent net accumulation of ions occurs. This suggests a tight coupling between ion uptake and cell growth in the undifferentiated cells near the epicotyl tip. If this ion accumulation is required primarily for the osmotic control of growth then it is reasonable that this component of transport would be inactivated in cells which have completed growth (e.g. in the region 1.5–3.5 cm back from the tip which was used in the experiments reported here). Perhaps excision allows reactivation of this ion transport mechanism in the absence of growth.

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