

## Involvement of Membrane Lipid Peroxidation in the Development of a Bacterially Induced Hypersensitive Reaction

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### ABSTRACT

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Membrane potential depolarization and increases in electrolyte leakage were observed during the development of a hypersensitive reaction in cucumber cotyledons infiltrated with the incompatible bacterial pathogen *Pseudomonas syringae* pv. *pisi* (*P. s. pisi*). These changes suggested an alteration of cellular membranes. Separation of the two membrane potential components suggested that both the passive component and the energy-dependent component were depolarized initially (after 2 and 3 hr of incubation) but that only the passive component showed continued depolarization throughout the 6-hr incubation period. We tested the possibility of lipid peroxidation as a mechanism by which the passive component of membrane potential was affected during a hypersensitive

reaction. Lipid peroxidation and electrolyte leakage were determined and compared in cotyledons treated with the following live or heat-killed bacteria: *P. s. pisi* (incompatible pathogen), *P. syringae* pv. *lachrymans* (compatible pathogen), and *P. fluorescens* (a saprophyte). Lipid peroxidation and electrolyte leakage increased significantly only in the combination producing a hypersensitive reaction. In cotyledons inoculated with the incompatible pathogen, lipid peroxidation increased concomitantly with membrane depolarization and before electrolyte leakage. Therefore, we suggest that lipid peroxidation is a key step in the membrane alteration that produces hypersensitive reaction symptoms (electrolyte leakage and tissue collapse).

During the development of a bacterially induced hypersensitive reaction (HR), affected tissues lose electrolytes, probably in response to irreversible alterations of cell membranes (3,6). Using electron microscopy, Goodman and Plurad (7) reported complete destruction of organelle membranes within 6 hr after infiltration of tobacco tissue with the incompatible pathogenic bacterium *Pseudomonas syringae* pv. *pisi* (*P. s. pisi*). Pavlovkin and Novacky (14), measuring membrane potential ( $E_m$ ) of cotton cotyledons infiltrated with the incompatible pathogen *P. syringae* pv. *tabaci*, observed a decline in  $E_m$  after 2 hr of incubation. Primarily because of function of the electrogenic pump, the energy-dependent component ( $E_p$ ) of  $E_m$  was unaffected, indicating that the observed change in  $E_m$  was the result of a decline of the diffusion potential ( $E_D$ ). This suggested alterations of membrane lipid as a possible mechanism.

Lipid peroxidation is a possible mechanism for alteration of the membrane lipid phase. Increases in lipid peroxidation have been reported during such processes as leaf senescence (5), ozone injury (13), anoxia (10), susceptibility to drought (4), herbicide injury (2), and wounding (15). An increase in lipid peroxidation could account for the depolarization of  $E_D$  (14) and the subsequent increase in membrane permeability (3,6) observed during the development of a bacterially induced HR (14). Therefore, we followed lipid peroxidation in plant cell membranes early in the development of a bacterially induced HR. We also followed electrolyte leakage and  $E_m$  during this same period. By comparing the timing of these three parameters, we tested the possibility that lipid peroxidation is an important step in the development of a bacterially induced HR. Preliminary results have been reported (11).

### MATERIALS AND METHODS

**Plants and bacteria.** Cucumber seedlings (*Cucumis sativa* L. 'Straight Eight,' Royal Seeds, Kansas City, MO) were grown in soil under fluorescent light for 8 days (16-hr photoperiod at 22–28 C). The bacteria used were: *P. s. pisi* (incompatible pathogen), *P. syringae* pv. *lachrymans* (*P. s. lachrymans*) (compatible pathogen),

and *P. fluorescens* (saprophyte). Bacteria were obtained from colonies derived from single cells and maintained on nutrient agar slants. The bacteria were transferred to nutrient broth and incubated at 25 C on a rotary shaker (150 revolutions  $\text{min}^{-1}$ ) for 24 hr to obtain cells in the log phase of growth. Bacteria were collected by centrifugation at 12,000  $g$  for 10 min, then resuspended in sterile deionized water. The cell concentration was adjusted with a spectrophotometer and verified by plate count. Heat-killed bacteria were obtained by autoclaving live bacteria (15 min, 121 C, 15 psi).

**Electrophysiology.** Cucumber cotyledons infiltrated with *P. s. pisi* ( $10^8$  cells  $\text{ml}^{-1}$ ) were held for 1 hr under fluorescent light ( $2 \times 15$  W) to allow dissipation of excess water from the tissue. A razor blade was used to cut segments ( $10 \times 15$  mm) from infiltrated and noninfiltrated cotyledons. The segments were aged for 1 hr by being floated on perfusion solution (1 mM KCl, 1 mM  $\text{Ca}(\text{NO}_3)_2$ , 1 mM  $\text{Na}_2\text{H}_2\text{PO}_4$ , 0.25 mM  $\text{MgSO}_4$ , pH 5.7) (9). The segments were placed in a 4-ml Plexiglas chamber through which perfusion solution flowed at the rate of 10  $\text{ml min}^{-1}$ . Micropipettes were inserted into the tissue with a micromanipulator and the aid of a horizontally mounted microscope.

Micropipettes were prepared as described by Novacky and Ullrich-Eberius (12) and filled with 3 M KCl. A reference salt bridge (3-cm section of polyethylene tubing) was filled with 3 M KCl in 2% agar. The two salt bridges were connected via Ag/AgCl electrodes to a WP Instruments model 701 electrometer-amplifier, a chart recorder, and a Tektronix dual-beam oscilloscope. Light was provided to the surface of the tissue segment by a quartz halogen bulb ( $118 \text{ W m}^{-2}$ ), using gooseneck fiber optics.

$E_D$  of the tissue was determined by measuring  $E_m$  when adenosine triphosphate (ATP) required for the function of the electrogenic pump was low. Low tissue ATP levels were obtained by bathing the segments in  $\text{N}_2$ -gas-saturated perfusion solution in the dark. Functionality of the electrogenic pump was tested by applying light under anoxic conditions and following the ability of the tissue to recover  $E_m$  to the original value measured under aerobic conditions in the dark.  $E_p$  was calculated by subtracting the mean of  $E_D$  from the mean of  $E_m$ .

**Lipid peroxidation.** Cucumber cotyledons (six per sample) received one of the following: infiltration with live bacteria ( $10^8$  cells  $\text{ml}^{-1}$ ), infiltration with heat-killed bacteria ( $10^8$  cells  $\text{ml}^{-1}$ ), or no treatment (controls). Cotyledons were infiltrated by introducing the needle of a hypodermic syringe through the lower epidermis.

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After 1.5, 3, 4.5, or 6 hr, samples were collected as disks (20/sample), using a paper punch (0.6 cm diam). Samples were transferred to 7-ml Teflon vials, liquid N<sub>2</sub> was added, and the samples were homogenized for 1 min with a Mikro-Dismembrator II (B. Braun Instruments). The powdered tissue was extracted with 0.2 M citrate-phosphate buffer (pH 6.5) containing 0.5% Triton X-100 by homogenization for 1 min with the Mikro-Dismembrator II. The brei was filtered through two layers of cheesecloth and centrifuged for 10 min at 20,000 g. The supernatant solution was retained as the crude extract. A 30- $\mu$ l aliquot of the crude extract from each sample was used for determination of protein concentration by the method of Bradford (1).

Lipid peroxidation was monitored by measuring the conversion of lipids to malondialdehyde (MDA) by the thiobarbituric acid reaction, with the minor modification of Dhindsa et al (5). A 200- $\mu$ l aliquot of crude extract was mixed with 1 ml of 0.1% trichloroacetic acid. The mixture was heated at 95 C for 30 min, then quickly cooled in an ice bath. After centrifugation (10,000 g for 10 min), the absorbance of the supernatant solution was measured at 532 nm. The value of nonspecific absorption at 600 nm was read and subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 mM<sup>-1</sup> cm<sup>-1</sup> (8) and expressed as nanomoles MDA mg<sup>-1</sup> protein.

Lipid peroxidation levels in cucumber cotyledons treated with each of the three bacterial species were measured in separate experiments with a minimum of four replications per experiment. To facilitate comparison of lipid peroxidation levels induced by treatment with live and heat-killed bacteria, the value obtained by live or heat-killed treatment was divided by the value obtained from a nontreated control, assayed in parallel, and expressed as a percentage of the nontreated control. Comparison of lipid peroxidation levels induced by heat-killed and live bacteria of the same species allowed the separation of possible changes in lipid peroxidation related to a bacterially induced host response from those resulting from a nonspecific wound response. The data from each incubation period were subjected to analysis of variance, and

statistically significant differences were determined using an orthogonal comparison ( $P > 0.05$ ).

**Electrolyte leakage.** As already described, cucumber cotyledons were infiltrated with live or heat-killed bacteria or sterile deionized water and the excess moisture allowed to evaporate. Fifty disks per sample were collected and floated on 50 ml of deionized water in a 125-ml Erlenmeyer flask. The flask was shaken (200 strokes min<sup>-1</sup>) with a Burrell wrist-action shaker. Fluorescent light (2  $\times$  15 W) was supplied throughout the incubation period. Aliquots (25 ml) were obtained at 60-min intervals, electrolyte leakage was measured as conductance ( $\mu$ mhos) with a YSI model 31 conductivity bridge, and the aliquots were returned to the incubation flask. At the completion of each experiment, the samples were autoclaved (10 min, 121 C, 15 psi) and total conductance measured. Electrolyte leakage was expressed as the mean of percent total conductance of bacterial infiltrated sample minus the mean of percent total conductance of water treatment.

TABLE I. Mean values of membrane potential ( $E_m$ ) and diffusion potential ( $E_D$ ) and calculated value of energy-dependent electrogenic pump component ( $E_p$ ) in noninfiltrated cucumber cotyledons and cucumber cotyledons infiltrated with *Pseudomonas syringae* pv. *lisi* ( $10^8$  cells ml<sup>-1</sup>)

Bacterial infiltration	Incubation (hr)	$E_m^a$ values (mV)		$E_D^b$ values (mV)		$E_p^c$ value (mV)
		Mean <sup>d</sup> $\pm$ SE		Mean <sup>d</sup> $\pm$ SE		
No	... <sup>e</sup>	-164	4.0	-73	1.2	-91
Yes	2	-137	9.7	-68	0.7	-69
Yes	3	-87	8.0	-55	1.5	-32
Yes	4	-80	5.5	-46	1.9	-34
Yes	6	-82	4.1	-38	1.9	-44

<sup>a</sup> Measured in the dark under aerobic conditions.

<sup>b</sup>  $E_m$  measured in the dark under anoxic conditions.

<sup>c</sup> Mean  $E_m$  minus mean  $E_D$ .

<sup>d</sup> Results of a minimum of three replicates.

<sup>e</sup> Tissue was floated on perfusion solution for 1 hr before measurements.

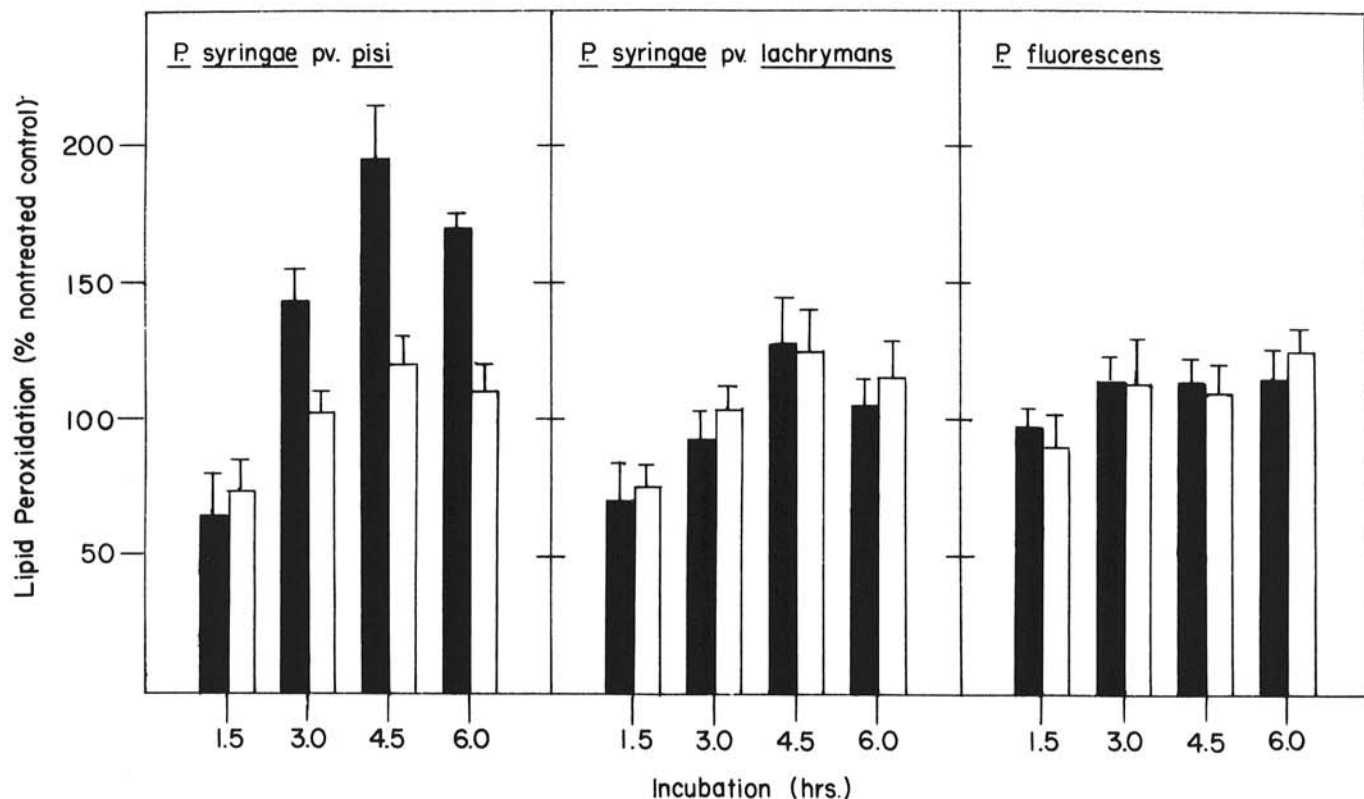


Fig. 1. Lipid peroxidation of cucumber cotyledons infiltrated with *P. s. pisi* (incompatible pathogen), *P. s. lachrymans* (compatible pathogen), or *P. fluorescens* (saprophyte), live or heat-killed ( $10^8$  cells ml<sup>-1</sup>), expressed as percent of nontreated controls (assayed in parallel). Solid bars = live bacterial treatments, open bars = heat-killed bacterial treatments. Mean values  $\pm$  SE are the results of a minimum of four replicates. Mean values  $\pm$  SE of combined nontreated controls were: 1.5 hr =  $2.63 \pm 0.19$ , 3.0 hr =  $2.50 \pm 0.16$ , 4.5 hr =  $2.04 \pm 0.13$ , and 6.0 hr =  $2.01 \pm 0.12$  nmols MDA mg<sup>-1</sup> protein.

## RESULTS

**Symptoms.** The first visible symptom of an HR, tissue collapse, was observable between 5 and 6 hr after infiltration of cotyledons with *P. s. pisi* (incompatible pathogen). Complete collapse of tissue infiltrated with *P. s. pisi* was observed between 10 and 12 hr after infiltration. The first visible symptom induced by the compatible pathogen (*P. s. lachrymans*) was similar (tissue collapse beginning at edges of cotyledon) to that observed in tissue infiltrated by the incompatible pathogen but was not observable until approximately 24 hr after infiltration. In the compatible combination, complete collapse of the cotyledonary tissue was not observed until after approximately 108 hr of incubation. No visible symptoms were observed (through 108 hr of incubation) in cotyledons infiltrated with the live saprophyte (*P. fluorescens*) or any of the heat-killed bacteria.

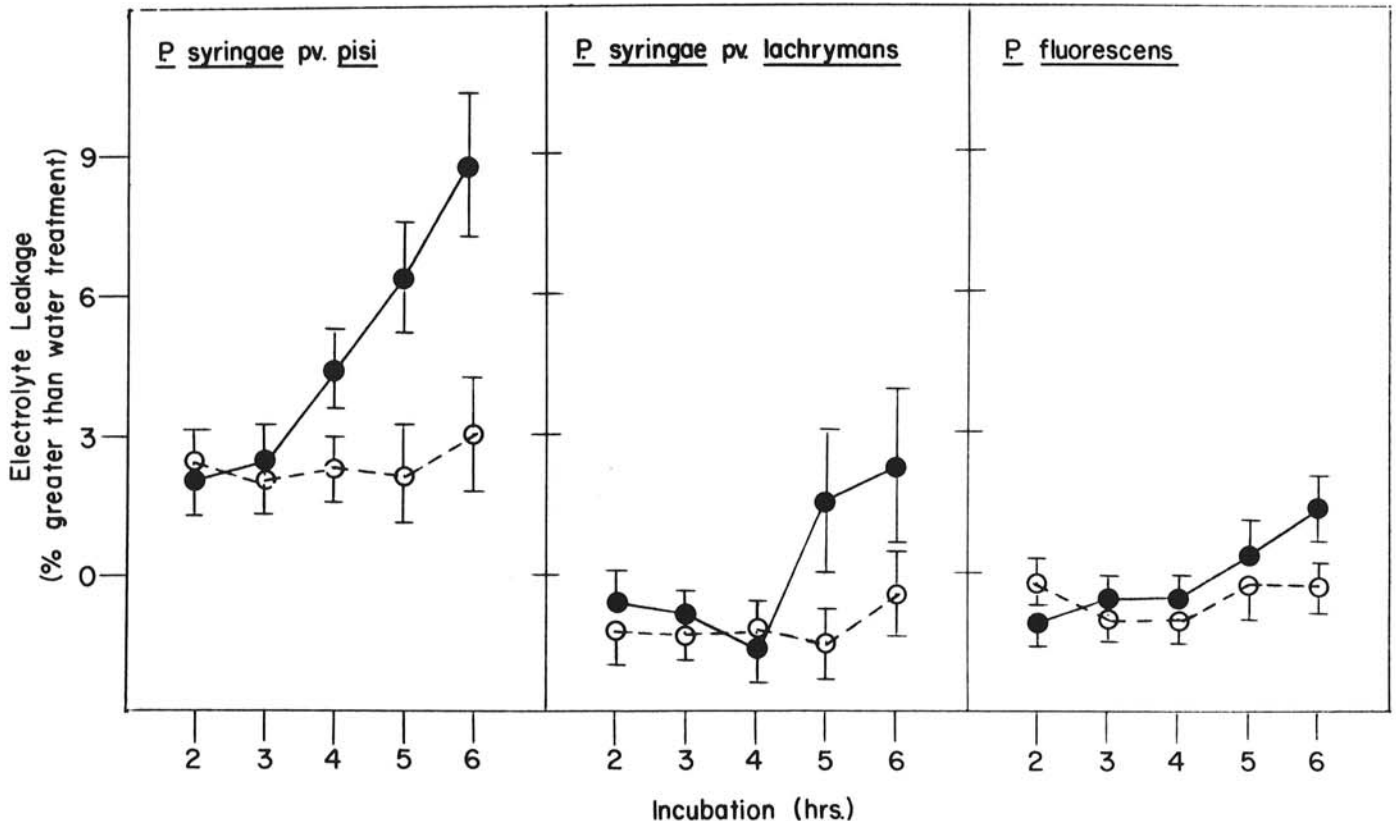
**Electrophysiology.** The electrogenic pump was functional in cucumber cotyledons infiltrated with *P. s. pisi* and in noninfiltrated control tissue. In noninfiltrated tissue,  $E_m$  was depolarized from  $-160$  mV in the dark to  $-70$  mV under anoxic conditions in the dark. Application of light allowed the repolarization (slight hyperpolarization) of the  $E_m$  to  $-168$  mV. Removal of light again depolarized membranes to  $-75$  mV, and reapplication of light repolarized them to  $-156$  mV. In tissue infiltrated with *P. s. pisi*, the ability to recover  $E_m$ , measured initially under aerobic conditions in the dark and after removal and application of light under anoxic conditions (test for functionality of electrogenic pump) throughout the 6-hr incubation period, was confirmed.

Comparison of the mean values of  $E_m$  and  $E_D$  and the values of  $E_p$  of noninfiltrated tissue and tissue infiltrated with *P. s. pisi* suggested that both components of  $E_m$  ( $E_D$  and  $E_p$ ) were depolarized in the treated tissue (Table 1). After 2 hr, the mean  $E_m$  of infiltrated

tissue was depolarized from  $-164$  mV in noninfiltrated tissue to  $-137$  mV in infiltrated tissue. A dramatic depolarization of  $E_m$  was observed between 2 and 3 hr after bacterial infiltration ( $-137$  mV to  $-87$  mV). Between 3 and 6 hr after infiltration, little change in  $E_m$  was observed. Two hours after bacterial infiltration, the mean value of  $E_D$  was  $-68$  mV and the value of  $E_p$  was  $-69$  mV, compared with a mean  $E_D$  value of  $-73$  mV and an  $E_p$  value of  $-91$  mV in noninfiltrated tissue. After 3 hr of incubation, the mean value of  $E_D$  was  $-55$  mV and the  $E_p$  value was  $-32$  mV in bacterially infiltrated tissue. The mean  $E_D$  of tissue infiltrated with *P. s. pisi* showed an increased depolarization during the entire incubation period, whereas  $E_p$ , after an initial dramatic depolarization, showed a slight repolarization after 4 and 6 hr of incubation.

**Lipid peroxidation.** Treatment with the live incompatible pathogen (*P. s. pisi*) induced significantly higher levels of lipid peroxidation than did treatment with the heat-killed incompatible pathogen (Fig. 1). After 3, 4.5, and 6 hr of incubation, lipid peroxidation was statistically significantly greater for live than for heat-killed *P. s. pisi* treatments. Lipid peroxidation levels induced by the live compatible pathogen (*P. s. lachrymans*) and the live saprophyte (*P. fluorescens*) were not statistically different from levels induced by their heat-killed bacterial counterparts at any of the sampling times (Fig. 1).

Lipid peroxidation levels induced by live or heat-killed incompatible or compatible pathogen treatments were similar after 1.5 hr of incubation and were lower than those of nontreated controls (Fig. 1). Lipid peroxidation levels induced by heat-killed incompatible or live or heat-killed compatible pathogen treatments were similar at all sampling times. After 3 hr, lipid peroxidation increased to the level of nontreated controls in these treatments. A small transient increase, to above nontreated control levels, was suggested after 4.5 hr, followed by a return to the control level after



**Fig. 2.** Electrolyte leakage of cucumber cotyledons infiltrated with water or *P. s. pisi* (incompatible pathogen), *P. s. lachrymans* (compatible pathogen), or *P. fluorescens* (saprophyte), live or heat-killed ( $10^8$  cells  $ml^{-1}$ ). Electrolyte leakage of bacteria-infiltrated cotyledons expressed as mean  $\pm$  SE of percent total conductance ( $\mu$ mhos) after autoclaving minus mean of percent total conductance after autoclaving of water-infiltrated control. Solid circles = live bacterial treatments, open circles = heat-killed bacterial treatments. Mean values of bacterially induced electrolyte leakage are the results of a minimum of five replicates. Mean values  $\pm$  SE of electrolyte leakage induced by water infiltration ( $n = 25$ ) were: 2 hr =  $15.3 \pm 0.3$ , 3 hr =  $17.9 \pm 0.2$ , 4 hr =  $19.3 \pm 0.3$ , 5 hr =  $20.7 \pm 0.4$ , 6 hr =  $21.1 \pm 0.4$ .

6 hr. Live and heat-killed saprophyte treatments induced lipid peroxidation levels similar to those of nontreated controls throughout the 6-hr incubation period (Fig. 1).

**Electrolyte leakage.** Throughout the 6-hr incubation period, infiltration of cotyledons with live or heat-killed *P. s. pisi* induced higher levels of electrolyte leakage than did infiltration with water (Fig. 2). Electrolyte leakage levels were similar for live and heat-killed *P. s. pisi* treatments after 2 and 3 hr of incubation, but dramatic increases were observed in cotyledons infiltrated with the live incompatible pathogen after 4, 5, and 6 hr of incubation. After 2, 3, and 4 hr of incubation, little difference in electrolyte leakage was observed between cotyledons infiltrated with the live or heat-killed compatible pathogen and those infiltrated with water (Fig. 2). After 5 and 6 hr of incubation, live compatible pathogen treatments induced slight increases in electrolyte leakage to levels greater than those induced by water infiltration of cotyledons. Throughout the 6-hr incubation period, levels of electrolyte leakage induced by the heat-killed compatible pathogen remained at or slightly below levels induced by water infiltration. Electrolyte leakage levels similar to those of the water-infiltrated controls were observed over the entire 6-hr incubation period for cotyledons infiltrated with the live or heat-killed saprophyte (Fig. 2).

## DISCUSSION

Significant increases in lipid peroxidation were observed only in the HR-producing combination (*P. s. pisi* × cucumber cotyledons). The initial, measurable increase was recorded after 3 hr of incubation. A measurable increase in electrolyte leakage was not observed until after 4 hr of incubation in this host/pathogen combination. These results suggest that increases in lipid peroxidation precede increases in electrolyte leakage. Therefore, we suggest that lipid peroxidation may be the mechanism by which membranes are altered and that these altered membranes in turn facilitate the loss of electrolytes observed during an HR.

With the compatible combination (*P. s. lachrymans* × cucumber cotyledons), increases in electrolyte leakage were observed but the timing differed from that of increases observed with the incompatible combination. Increases to levels greater than those observed with the water infiltration treatment were not observed until after 5 hr of incubation. Between 2 and 4 hr of incubation, levels of electrolyte leakage induced by *P. s. lachrymans* actually decreased to below those of the water-infiltrated controls. In addition, electrolyte leakage levels induced by treatment with the heat-killed compatible pathogen were lower than those induced by the water-infiltrated controls throughout the 6-hr incubation period. These results suggest that the presence of *P. s. lachrymans* (live or heat-killed) may induce a host cell reaction(s) that affects retention of electrolytes. The slight increases in electrolyte leakage seen after 5 and 6 hr of incubation, without concomitant increases in lipid peroxidation, may be due to the compatible pathogen inducing damage to host cells by some mechanism other than lipid peroxidation.

Pavlovkin and Novacky (14) recently reported that membranes were depolarized during the development of a bacterially induced HR in cotton. They observed that the ATP-requiring electrogenic pump was functional throughout a 10-hr incubation period, that depolarization of  $E_m$  could be measured as early as 2 hr after bacterial inoculation, and that depolarization of  $E_D$  increased throughout the 10-hr incubation period. We obtained similar results using their procedure (14) and our HR-producing combination. We observed that the electrogenic pump was functional throughout a 6-hr incubation period, that depolarization of  $E_m$  was observed after 2 hr of incubation, and that depolarization of  $E_D$  increased throughout the 6-hr incubation period. The similarity of the results obtained from these two different HR-producing combinations suggests that the observed changes in membrane potential during the early stages of an HR may be general phenomena.

Because of the requirement for dissipation of excess water from cotyledonary tissue and aging of tissue on perfusion solution, we could not measure  $E_m$  in bacteria-infiltrated tissue before 2 hr of

incubation. Thus, we can only suggest that initial depolarization of  $E_m$  occurred by the end of 2 hr of incubation. The largest depolarization of  $E_m$  was observed between 2 and 3 hr after bacterial infiltration. Separation of the two  $E_m$  components suggested that both the electrogenic pump component and the diffusion component were dramatically affected after 2 and 3 hr of incubation. The  $E_p$  was actually depolarized more than was the  $E_D$ . Therefore, the site of initial effect on the plasma membrane may be the electrogenic pump. This effect on the electrogenic pump is probably not the membrane alteration responsible for subsequent electrolyte leakage, because after 4 and 6 hr of incubation (when electrolyte leakage can first be observed), the  $E_p$  is being repolarized while the  $E_D$  is still being depolarized. In addition, the electrogenic pump was found to be functional throughout the 6-hr incubation period. In contrast, depolarization of  $E_D$  closely paralleled both the initial and the continued increases of lipid peroxidation. Therefore, it seems likely that the dramatic and continued depolarization of  $E_D$  reflects membrane alteration responsible for subsequent electrolyte leakage.

In view of the foregoing results, we propose the sequence of events during the development of a bacterially induced HR may be as follows: Lipid peroxidation of host membranes (including plasma membrane) is initiated by some unknown mechanism and increases to a level that alters the membrane lipid phase. This alteration of the lipid phase of the plasma membrane can be measured initially as depolarization of  $E_m$ ,  $E_D$ , and possibly  $E_p$ . As lipid peroxidation continues, alteration of the membrane lipid phase is expressed as electrolyte leakage and continued depolarization of  $E_D$ . The continued in situ loss of electrolytes into the intercellular spaces increases the osmolarity outside the cells, and a flow of water from the cells follows. The enhancement of osmolarity of the intercellular space is supported by water evaporation through the epidermis. Eventually, tissue cells become plasmolyzed and the visible symptom of an HR (tissue collapse) can be observed.

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