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STUDIES ON THE CONTROL OF CELL WALL EXTENSION

Yearly Progress Report

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MASTER

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PROGRESS REPORT, RLO-2225-T19-52

Contract: EY-76-S-06-2225-T19

Principal Investigator: Robert E. Cleland, Professor of Botany
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I. Introduction:

The research in this laboratory is centered around the question as to how plant cell enlargement is controlled and regulated at the cellular level. Cell enlargement is influenced by many factors, but most of them act indirectly. For example, growth is altered by such environmental factors as light and temperature, and by internal factors such as the genetic complement of the cells, yet none of these directly control cell enlargement. The metabolism of cells, including the production of ATP and the synthesis of RNA, can have a major impact on cell enlargement, but again the effects are indirect. Theoretical considerations, and the research of Lockhart, Green and myself have shown that the growth rate (dV/dt) of cells can be defined in terms of five parameters:

$$dv/dt = \frac{L'p \cdot f(dW/dt \cdot WLC) \cdot (\pi_c - \pi_o - Y)}{L'p + f(dW/dt \cdot WLC)}$$

where $L'p$ is the hydraulic conductivity of the tissue, dW/dt is the rate of export from the cell to the wall of a wall loosening factor, WLC is the capacity of the wall to be loosened in response to the wall loosening factor, π_c and π_o are the osmotic potential of the cells and outside solution, respectively, and Y is the yield threshold for wall expansion. When a cell is

induced to expand, the expansion must occur by a change in one or more of these five parameters. Likewise, when growth ceases it must because of changes in these parameters. The basic goal of my research is to assess each of these parameters under conditions where growth is being enhanced or inhibited, with the purpose of explaining at the cellular level just how the growth is being controlled.

In the past most of our work has been concentrated on one single situation; the induction of cell elongation in the oat coleoptile by the hormone auxin. Addition of auxin causes the growth rate of coleoptile sections to increase by at least 5-fold after a lag of only about 10 minutes, making it one of the fastest hormonal responses known in plants. Our research during the 60's provided strong evidence that the effect of auxin is to increase the extensibility of the cell walls. All the indications were that auxin increased the export of a wall loosening factor from the cells to the walls (dW/dt) but the identity of W was not known. Then in 1970-71, Hager and coworkers in Germany, and Rayle and myself proposed that the wall loosening factor was simply hydrogen ions. The "acid-growth" theory which Hager and ourselves formulated states that auxin causes the cells to excrete protons, and that the resulting lowered pH of the walls activates some polysaccharide degrading enzymes in the walls, with the result that the wall becomes loosened and cell expansion takes place. It should be noted that this mechanism is similar to the one involved in protein digestion in the stomach, where the hormone gastrin induces the excretion of protons (HCl) into the stomach, and the lowered pH activates the enzyme pepsin. In the years following the formulations of the acid-growth theory we were principally involved in obtaining evidence as to whether the theory was correct

or not. We accumulated an impressive amount of data which seemed to indicate that protons are the wall loosening factor in auxin-induced growth of stem and coleoptile cells. However, this conclusion was based on an assumption; namely, that the cuticular layer surrounding the tissues is only slightly permeable to protons and buffers unless the permeability is enhanced by scarification or by removing the epidermal layers. While this seemed like a reasonable assumption, it had never been tested. One part of our research was therefore directed towards measuring the proton permeability of soybean and sunflower hypocotyl cuticular layers, both intact and after scarification. We have demonstrated the low permeability of the cuticular layers to protons, and have shown that scarification greatly enhances this permeability. Details are given in Section IIA. As a result of these studies we feel that we can conclude with confidence that protons are a necessary intermediate in auxin-induced cell elongation, and are the wall loosening factor.

We have now been directing our attention towards the other four factors which might control the rate of cell elongation. During the past year we have initiated a major study of the control of the control of the osmotic potential of the cell, π_c, and its relation to cell elongation. As long as Lp is sufficiently high that water permeability is not limiting growth, and as long as the cells are growing in a solution of constant π_o, the turgor pressure of the cell will be proportional to $-\pi_c$. As a cell takes up water during growth, the $-\pi_c$ of the cell decreases and the turgor pressure decreases along with it. Since the growth rate is proportional to the turgor pressure in excess of Y, small decrease in the turgor pressure can cause a large decrease in the growth rate.

When a cell expands during growth, and takes up water, the growth rate will fall to zero due to this dilution of the osmotic concentration unless osmoregulation takes place. Osmoregulation is the generation of new osmotic solutes within a cell, either by uptake of salts or sugars, or by production of solutes by photosynthesis or by solubilization of insoluble materials such as starch. If osmoregulation matches water uptake, the π_c and turgor pressure will remain constant and thus the growth rate can remain constant, but if osmoregulation exceeds or lags behind water uptake, the π_c will change and the growth will also change.

Osmoregulation has been most extensively studied in marine algae such as Valonia, Ochromonas and Dunaliella. In these cells fluxes of K^+ or production and breakdown of organic compounds occur in response to changes in turgor pressure. Osmoregulation in higher plants has received far less attention, however. It is known that coleoptile and stem sections take up osmotic solutes from the external solution, and that the uptake is enhanced during auxin-induced growth. But the relation between growth and osmoregulation, the control of the uptake, and even the timing of the osmoregulation remained obscure.

We have therefore initiated a study of osmoregulation in Avena coleoptiles. We have shown that there are two components to this osmoregulation. Cells take up sucrose even in the absence of any water uptake. This uptake is not regulated by turgor pressure, as the uptake is essentially the same whether the cells are fully turgid or have had their turgor reduced with osmotic agents such as mannitol. In addition, whenever there is growth -- i.e., water uptake -- there is additional sucrose uptake which is proportional to the growth. This uptake is not induced by auxin, as it fails to occur if the auxin-induced growth is blocked by calcium ions or mannitol, but does occur

when growth is induced by the fungal toxin fusicoccin rather than auxin. Details of this study will be found in Section IIB.

The other factor we have been examining is the wall loosening capacity; i.e., the ability of cells to undergo wall loosening when placed in an acidic environment. To measure the WLC, tissues are incubated under various conditions, then frozen-thawed, and placed under constant tension (e.g. 20 g) in a pH 7 buffer. Upon change of the buffer to one at pH 3 the tissue undergoes a certain amount of acid-induced extension. This extension starts at a rapid rate, but then the rate decreases continuously over a period of several hours. In measuring the WLC the problem is what to measure and when to measure it. Two years ago Mark Tepfer, who was then a graduate student in my lab, showed that the extension curve fit the first-order rate equation: $l = b - ae^{-kt}$ where l is the length, t is time, and b, a and k are constants. The shape of the extension curve, the amount of extension, and its rapidity are dependent upon the values of the two constants a and k. The value of a determines the total amount of extension which will occur, while k describes the rapidity.

During the past year we have been analyzing acid-induced extension curves for their a and k values, using the computer to give us the best fit. We have shown that as the pH is decreased, the principal effect is an increase in a. An increase in temperature, on the other hand, causes an increase in k with no real change in a. An increase in tension gives unexpected results; as the tension increases there is first a decrease in k without a change in a, followed by a large increase in a with no further decrease in k. It is apparent from these results that in order for acid-growth curves to be meaningful they must be analyzed via the computer and the extension characterized by a and k values. Details of this research will be found in section IIC.

II. Specific Research Projects

A. Proton Permiability of Plant Cuticles

Sheryl Dryer, Virginia Seymour and I have been examining the permeability of cuticles to protons. The basic procedure we have evolved is as follows. Epidermal strips are peeled off of light or dark-grown sunflower or soybean hypocotyls. They are frozen-thawed to disrupt the remaining cells, and are then glued over a 1 x 3 mm opening in the bottom of a plastic beaker cup. The inner side of the strip is the side which is actually glued to the cup. A 70 µl drop of 0.01 M KCl, pH 6.4 is placed on the inner surface and a combination pH electrode is lowered directly onto the drop. This whole apparatus is then placed onto the surface of a 0.01 M KCl solution at pH 3.0, and the movement of protons from the lower, outside solution across the cuticle and epidermal walls into the inner solution is measured continuously.

The results of this study have been prepared for publication. The manuscript, which has been submitted to Planta, is appended. The basic conclusions which we reached are as follows: 1) the proton permeability of unabraded cuticular layers is extremely low; 2) if the cuticle is abraded there is a dramatic increase in the proton permeability; 3) over long periods of time protons do cross the unabraded cuticle at a rate similar to that reported for other cuticles for K^+.

B. The Control of Osmoregulation in Avena Coleoptiles

The purpose of this study is to determine how osmoregulation is controlled in Avena coleoptiles and how it relates to cell elongation. This work has been primarily carried out by Tom Stevenson.

The technique consists of incubating 14 mm sections (deleafed) of Avena coleoptiles in a solution containing either 2% sucrose or 30 mM NaCl, and after

the desired length of time, giving the sections a rapid chase in cold water followed by carefully drying the sections and then freezing them. Then one of two techniques was used. In the first, the sections were thawed, the cytoplasm was squeezed out, and its osmotic concentration (OC) was determined using an Advanced Cryoscopic Osmometer. Alternatively, sections were thawed in the sample chamber of a Wescor Vapor Pressure Microosmometer and the OC was determined. The latter technique has the advantage of using much less material, meaning that more samples could be run per experiment. Its disadvantage is that the reading includes both the OC and the matrix potential, but we have determined that the matrix potential is so insignificant that it can be ignored.

Published values for the OC of Avena coleoptiles have placed it around 400 mOs, but when we first measured it we obtained values closer to 260 mOs. Because we were worried that our technique might be introducing a major error, we have remeasured OC in two different ways. First, we incubated sections in a graded series of mannitol solutions and then examined them for plasmolysis under the microscope. Plasmolysis could be detected in 0.3 M mannitol. Secondly we incubated sections in a graded series of mannitol concentrations containing ^{14}C-mannitol, and then determined the ^{14}C content within the tissue. The ^{14}C content remains low as long as the cells are not plasmolyzed, but rises rapidly as plasmolysis increases. From the resulting curves it was apparent that plasmolysis occurred whenever the OC of the solution exceeded about 260 mOs. Thus we conclude that the OC of our Avena coleoptiles was, indeed, in the range of 260 mOs.

We then determined that coleoptiles can osmoregulate using either exogenous sugars or salts as the osmotic agent. The optimum sucrose concentration is 2%; in its presence auxin-induced growth continues at a constant rate for

at least 20 hours. NaCl has proven to be a better osmotic agent than KCl, to our surprise. The optimum concentration for NaCl is 30 mM, and again, growth continues at a constant rate for many hours in its presence. In the absence of auxin coleoptile sections take up sufficient osmotic solutes from either sucrose of NaCl to increase their OC to 310-320 mOs in 10 hrs; the total osmotic solutes increases by 25-30% during this time. In the presence of auxin the sections grow about 40% during the 10 hrs, and take up additional osmotic solutes, but since the uptake of osmotic solutes just keeps pace with the water uptake the OC remains constant at about 260 mOs. Sections growing in response to auxin, but without any absorbable osmotic solute, decrease in OC, and the growth rate falls off after 4-6 hours.

The increased osmoregulation which occurs in the presence of auxin might be a response to auxin or to the auxin-induced growth. It would appear to be in response to the growth, since when the growth is inhibited by calcium ions (Table 2) or by osmotica such as mannitol or PEG, the effect of auxin on osmoregulation disappears. If we induce elongation with the fungal toxin fusicoccin instead of auxin, we get osmoregulation similar to that induced by auxin.

The osmoregulation can be divided into two components. In the absence of any growth considerable solute uptake occurs. Then, in addition, there is extra uptake which is proportional to the amount of growth (Fig. 1). This is shown by varying the growth rate through variation in the auxin concentration. There may also be a third component, which begins only 4-5 hours after addition of auxin. When sections are incubated in 2% sucrose and auxin, the OC of the sections remains virtually constant for about 4 hours. Then it begins to rise (Fig. 2) and the increase in OC continues for at least

another 10 hours. This suggests that some mechanism for enhanced uptake is induced, but only after a considerable lag.

What regulates osmoregulation? In marine algae it would appear to be the turgor pressure. But in the Avena coleoptile turgor pressure does not seem to be important. If the turgor pressure is decreased by addition of impermeant osmotica such as mannitol or PEG, the rate of osmotic solute uptake is hardly affected. Sections incubated in sucrose without auxin continue to take up osmotic solutes and thus increase their turgor pressure throughout a 24 hour period, indicating that increased turgor does not shut off osmotic solute uptake as it does in the algae. Further work is needed before we can determine the control mechanism for osmoregulation in coleoptiles.

All of the previous experiments have been conducted with sections which had intact cuticular layers, since most of the growth data have come from similar sections. But the cuticle is a barrier to the uptake of sugars; this is shown by comparing the increase in osmotic solutes between intact and peeled coleoptiles (i.e., sections whose epidermal layer has been peeled off). As can be seen from Table 3, peeled sections take up considerably more osmotic solutes. This suggests that in intact sections the uptake of sugars is primarily through the cut ends, and that when a growing section has a constant OC, it does not mean that all cells in the section also have a constant OC. To test this we have marked 14 mm sections into 3 areas with ink, allowed them to grow for two hours in the presence of auxin and sucrose, and then determined the growth and the OC of each of the 3 areas (Table 4). It can be seen that the apical and basal areas, which contain the two cut ends, both increase in osmotic solutes and in OC, while the center area takes up no solutes and has a decreasing OC. We were surprised

to find, however, that the apical and center areas showed the same growth. Furthermore, their growth as part of the 14 mm section was the same as when they were first separated into 5 mm sections. Thus the apical and center areas are growing at the same rate, even though in one the turgor pressure is increasing and in the other it is decreasing. It is apparent that we must carry out more extensive investigations into this problem.

In conclusion, we have shown that oat coleoptiles can use either sugars or salts for osmoregulation, that the uptake into intact sections consists of at least two components, one of which is proportional to the growth, and that turgor is not the controlling factor in determining the rate of osmoregulation.

C. An analysis of the acid-extension curves

Dr. Mark Tepfer and I have been carrying out an analysis of the acid-extension curves for Avena coleoptiles. We have known that the acid-induced extension is influenced by the pH of the external solution, by the tension applied to the walls and by the temperature, among other factors. We have shown previously that the extension curve fits the equation:

$$l = b - ae^{-kt}$$

where l is the length, and a and k are constants. We have known that each of the factors mentioned above could modify the extension by altering a, k, or both. We have undertaken this analysis in order to clarify the situation.

Frozen-thawed Avena coleoptiles were placed in our constant-stress apparatus under 5-20 g stress and in the presence of a pH 7 K-phosphate buffer. After 30 minutes the solution was changed to a more acidic one, and the extension was recorded. Extension values were measured from the chart at a series of time intervals, and the computer was used to fit the equation

above to the data. The computer gives us the best values for a and k, and an error-mean-square value which tells us how well the data fit the equation. This is a slow procedure, but since we have no microcomputer of our own, it is the only available method. The a and k values are then averaged from a series of runs.

The effect of tension on the acid-extension is shown in Fig. 3. As the tension is increased, two effects are noted. First, a (which is a measure of the total extension) increases after the tension reaches 10 g. It would appear that acid-extension has a yield stress for extension, just as does creep and in vivo auxin-induced growth. The second effect, which was unexpected, is a large decrease in k as the tension is increased. This means that with greater tension, the reaction occurs at a slower rate. This effect needs to be examined in greater detail.

At pH 7 the extension curve actually fits a log-time equation better than the exponential equation. As a result the values for both a and k in Table 5 are probably too high. As the pH is decreased, a increases, but there is essentially no change in k. These results are what we would have predicted, but the analysis needs to be extended now to situations where a greater stress is used. As the temperature is raised from 15^{o} to 25^{o} and then 35^{o}, there is an increase in k with no change in a. Again, this is what we would have predicted.

This analysis must be expanded before we can reach any final conclusions. But it is already apparent that acid-extension can only be characterized by fitting the extension to the equation, and determining a and k values. When comparing acid-extension between two situations one must be able to compare both of these values.

III. Personnel Connected with this Research

Dr. Robert E. Cleland, Principal Investigator

Collaborators: Dr. B. Rubinstein, Professor of Botany, University of Massachusetts, Amherst, MA

Dr. Mark Tepfer, Postdoctoral Fellow, Botany Department, University of British Columbia, Vancouver, B.C., Canada

Graduate Students: Tom Stevenson

Virginia Seymour

Sara Mandel

Undergraduate Student: Sheryl Dryer (until June)

Research Technician: Sheryl Dryer (after June)

IV. Publications Resulting from this Research

Research Papers:

Goldsmith, M. H. M. & R. E. Cleland. 1978. The contribution of tonoplast and plasma membrane to the electrical properties of a higher-plant cell. Planta 143, 261-265.

Tepfer, M. & R. E. Cleland. 1979. Comparison of acid-induced cell wall loosening in Valonia ventricosa and in oat coleoptiles. Plant Physiol. 63, 898-902.

Bates, G. W. & R. E. Cleland. Protein synthesis and auxin-induced growth: inhibitor studies. Planta 145, 437-442.

Abstracts:

Cleland, R. E. 1979. Auxin and H^+-excretion: the state of our knowledge. X Internat. Conference on Plant Growth Substances, Madison, WI.

V. Seminars Given on this Research

Botany Department, North Carolina State University, Raleigh. November 1978

Biology Department, W. Washington St. University, Bellingham. April 1979

Table 1. Osmoregulation from sucrose and NaCl solutions. Sections were incubated for 10 hours in 2% sucrose or 30 mM NaCl, ± 10 μM IAA. Length, the osmotic concentration (OC) and the increase in osmotic solutes (ΔOS) were then determined.

Conditions	ΔL(%)	OC(M)	ΔOS(%)
Initial	-	.263	-
2% sucrose, -IAA	6	.322	+29
" , +IAA	41	.270	+45
30 mM NaCl, -IAA	6	.312	+26
" , +IAA	37	.259	+35
No solutes, +IAA	26	.167	-20

Table 2. Inhibition of auxin-induced increase in osmoregulation by inhibition of growth. Sections were incubated 10 hours in 2% sucrose, ± 10 μM IAA and with or without 20 mM $CaCl_2$.

Conditions	ΔL(%)	OC(M)	ΔOS(%)
Initial	-	.257	-
No Ca, -IAA	7	.299	+24
" , +IAA	41	.263	44
20 mM $CaCl_2$, -IAA	2	.324	29
" , +IAA	3	.319	28

Table 3. Effect of removing epidermis on osmoregulation. Intact sections from which the epidermal layers had been peeled were incubated 6 hours in 2% sucrose, ± 10 μM IAA and then length, OC and ΔOS were determined.

Conditions		ΔL(%)	OC(M)	ΔOS(%)
Intact:	initial	-	.259	-
	6 hr, - IAA	5	.299	21
	6 hr, + IAA	31	.265	34
Peeled:	initial	-	.262	-
	6 hr, -IAA	2	.342	33
	6 hr, +IAA	9	.367	53

Table 4. Growth and osmoregulation in areas of a coleoptile section. 14 mm coleoptile sections were marked into 3 areas with ink, then incubated for 2 hours in 2% sucrose + 10 μM IAA. Each area was then measured, separated, and its OC was determined.

Area	Initial OC	OC, 2 hr (M)	ΔL(%)	ΔOS(%)
Apex	.258	.266	11	15
Middle	.269	.244	12	1
Base	.268	.278	4	8

Table 5. Effect of pH on parameters governing acid-induced extension. Sections were frozen-thawed, then incubated under 10 g tension in a pH 7 solution. The pH was changed to that indicated, and the resulting extension curve was fitted to the exponential equation with a computer. Each value is the average of 8 runs.

pH	a (units)	k (min^{-1})
7.0	12	.0656
5.0	18	.0354
4.0	31	.0350
3.6	29	.0341
3.0	31	.0388

FIGURE LEGENDS

Fig. 1 Relation between extension and osmoregulation. Sections were incubated for 6 hours in 2% sucrose with 10^{-8} to 10^{-5} M IAA. Amount of extension and the OC were determined for each group of sections, and the increase in osmotic solutes was then calculated.

Fig. 2 Time course of change in OC in auxin-treated sections. Groups of coleoptile sections were incubated in 2% sucrose + 10 μM IAA, and after varying times their OC was determined.

Fig. 3 Effect of tension on the parameters governing acid-extension. Frozen-thawed coleoptile sections were incubated under 5-20 g tension at pH 7, then the pH was changed to 4.0 and the extension curve was measured. The data were then fitted to the exponential equation with a computer to give values of a and k.

Fig 1

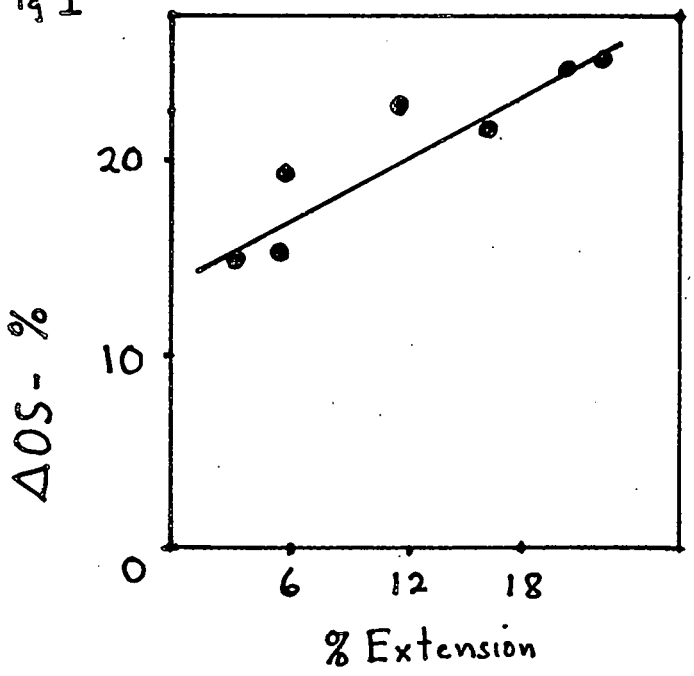


Fig 2

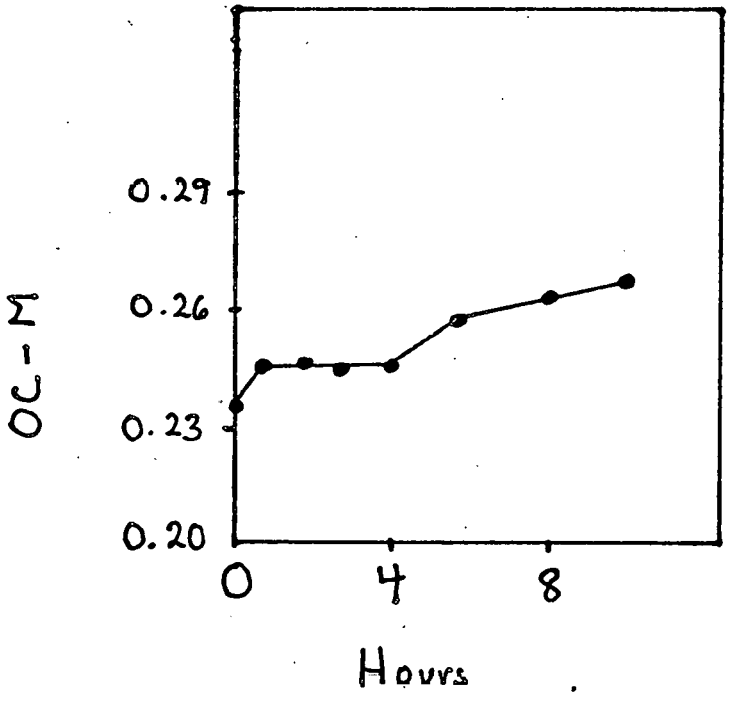
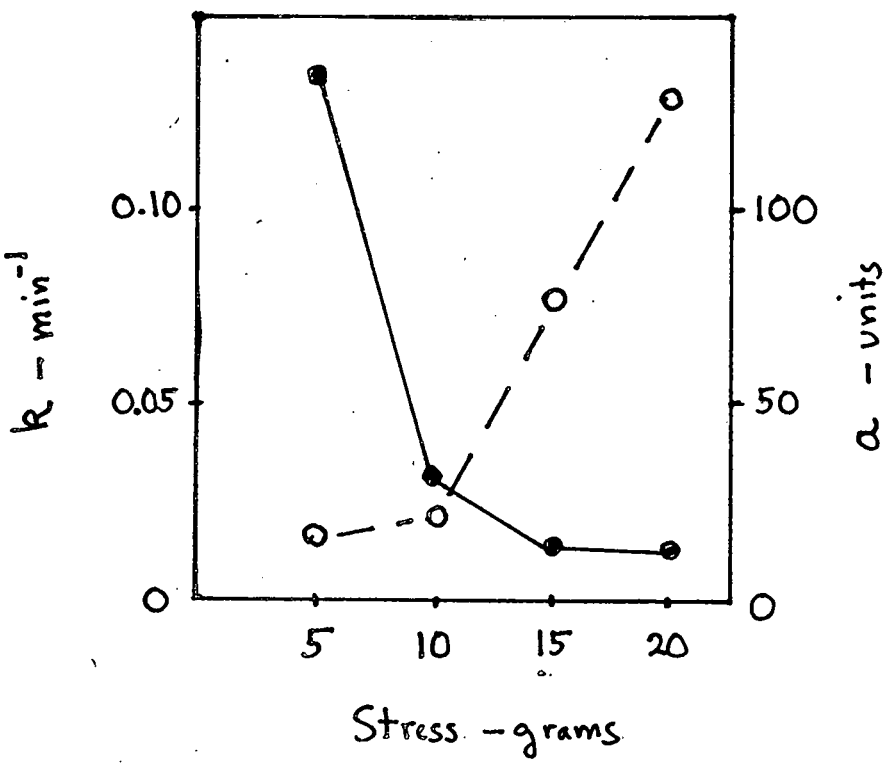


Fig 3



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THE IMPERMEABILITY OF PLANT CUTICLES TO PROTONS, AND ITS RELEVANCE TO THE ACID-GROWTH THEORY

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Abstract. The impermeability of plant cuticles to protons has been demonstrated by measuring the ability of protons to cross frozen-thawed epidermal strips of sunflower and soybean hypocotyls, and isolated cuticles of Berberis leaves and tomato fruit. Diffusion of protons across the cuticle is extremely slow unless the permeability has been enhanced by scrubbing (scarification of the surface with emory) or by removing cuticular waxes. The permeability is greater when acetic acid is used as the acid as compared with HCl. Scanning electron micrographs show that scrubbing disrupts the integrity of the cuticle. The impermeability of the cuticle to protons explains many of the difficulities that have been experienced in measuring auxin-induced proton excretion from stem tissues.

Key Words: Acid growth - Cuticle - Proton permeability - Scrubbing.

Introduction

The aerial portions of all higher plants are covered by a cuticle, which is believed to act as a barrier to the entry and exit of materials from tissues (Martin and Juniper, 1970). The ability of the cuticle to limit movement of cations such as K^+ (Yamada et al., 1964; McFarlane and Berry, 1974), organic substances (Darlington and Cirulis, 1963) and water (Schonherr, 1976) has been established, but the permeability of a cuticle to protons has apparently never been determined. Knowledge of the proton permeability of the cuticle is of particular importance in regard to the acid-growth theory of auxin-induced growth (Rayle and Cleland, 1977). This theory states that auxin causes cells to excrete protons into the wall solution where the resulting lowered pH activates wall loosening enzymes. If sections are floated on a medium, and if the cuticle is permeable to protons, any excreted protons would be expected to diffuse out into the external medium and cause a marked decrease in the pH of the solution. Such an acidification of the external medium has been difficult to demonstrate (Penny et al., 1975; Parrish and Davies, 1977; Vanderhoef et al., 1977) unless the cuticle is scarified (Mentze et al., 1977; Cleland and Rayle, 1978) or removed (Cleland, 1973; Rayle, 1973); this led to the suggestion (Cleland, 1973; Rayle, 1973; Rayle and Cleland, 1977) that the cuticle is an effective barrier to the diffusion of protons. However, as pointed out by Vanderhoef et al. (1977) this assumption has never been tested. In this paper we demonstrate the relative impermeability of plant cuticles to the movement of protons.

Materials and Methods

Plant Material

Seedlings of sunflower (Helianthus annuus L., var. Russian Mammoth) were grown for 5 days at 25 °C under constant illumination (400 μE m^{-2} sec^{-1}). Epidermal strips, 3-4 by 15-25 mm, were then peeled from the upper, growing regions of the hypocotyl with fine forceps. The strips were placed on glass slides, frozen on dry ice, and stored frozen and wrapped in foil until use. Seedlings of soybean (Glycine max L. var. Wayne) were grown at 25°C in very dim red light (<10^{-2} μE m^{-2} sec^{-1}) or under constant white illumination (400 μE m^{-2} sec^{-1}), and strips were then isolated in the same manner.

The cuticle of some seedlings was abraded by rubbing the hypocotyl 5-10 times with a slurry of emory powder (American Optical, Grade 120) before isolation of the epidermal strips. Other seedlings were wiped twice with a 3:1 mixture of chloroform:ethanol to remove some of the cuticular waxes.

Leaves of Berberis were selected from a plant growing outside the laboratory. To isolate the cuticle, leaf pieces were infiltrated and incubated for at least 2 days with a solution containing 8% pectinase (ICN #102533, Cleveland, OH) and 0.8% cellulase (ICN #101308) in 0.1 M Na-acetate buffer, pH 3.7. The upper epidermis was then lifted off the piece with fine forceps, washed well with water, and stored frozen until use. Enzymatically-isolated tomato fruit cuticle was a gift of Dr. M. A. Bukovac.

Proton Permeability Measurements

To measure the relative permeability of the cuticle to protons we used the apparatus shown in Fig. 1. The epidermal

strips or isolated cuticles were thawed and then glued across a 1x6 mm opening cut in the bottom of a 5 ml plastic beaker cup (#13915-985, VWR Scientific, Seattle); the inner surface of the strip was against the lower surface of the cup. A 100 µl drop of 10 mM KCl, pH 6.5, was placed over the opening against the inner surface of the strip and a combination pH electrode (Ingold #6020, Cambridge, MA) was inserted into the drop. This assembly was lowered into a beaker containing 5 ml of 10 mM KCl, pH 3.0, so that the external surface of the cuticle was in contact with a pH 3 solution while the internal surface was in contact with a pH 6.5 solution. The pH of the drop on the inner surface of the cuticle was then continuously recorded.

Some epidermal strips were preincubated for 3 hrs. on a 0.2 mg/ml solution of Pronase in 10 mM Tris-HCl buffer, pH 7.5, in order to remove proteins associated with the cell walls adhering to the inner cuticular surface. The strips were then washed well with water before use.

It should be pointed out that considerable care is needed in insure reproducibility. The scrubbing process is difficult to quantify with the result that the rapidity with which protons cross scrubbed strips shows some variation. The results presented here have been selected as representative of the data obtained from a large number of trials.

Scanning Electron Microscopy

Thawed epidermal peels were air dried, coated with gold-palladium, and viewed in a JOEL Model JSM-U3 scanning electron microscope.

Results

The ability of protons to diffuse from the lower (pH 3.0) solution into the upper (pH 6.5) solution in our apparatus was first tested using dialysis membrane as the barrier (Fig. 2). There is a rapid diffusion of protons, resulting in a drop in the pH of the upper solution to 4.0 within 10 minutes. Complete equilibration of protons takes more than an hour. When $\log((H^+) - (H^+)_i)$ is plotted as a function of time, a straight line is obtained, as expected for a diffusion process (Fig. 2, insert).

When scrubbed strips from light-grown sunflower hypocotyls are used as the barrier, the movement of protons across the epidermal strip is as rapid is it is across dialysis membrane (Fig. 2). However, when unscrubbed strips are used, the diffusion of protons is greatly impeded. After 10 min. the pH of the solution on the inner side of the strip has decreased less than 0.1 pH unit, and even after an hour the pH is still well above 6. The impermeability of the cuticle to protons is independent of the direction of proton movement, as a similar lack of proton diffusion across unscrubbed strips is obtained when the strip is reversed so that the acid is against the inner surface (data not shown).

With longer periods of time, proton diffusion across unscrubbed epidermal strips is detected (Fig. 3). Ater a lag which varies between 50 and 150 minutes the pH of the upper solution begins to drop, and in the example shown in Fig. 3 the pH had reached 3.76 after 900 min. When $-\log((H^+) - (H^+)_i)$ is plotted as a function of time, a straight line is obtained only after a long lag. Extrapolation of the linear portion of this curve back to the abscissa gives intercept values ranging from

350 to 500 min. The linear portion of the curve can be used to calculate a relative permeability constant for protons, using the equation (McFarlane and Berry, 1974)

$$dn/dt = k \cdot a \cdot \Delta C$$

where dn/dt is the moles of protons moving across the barrier in time t, a is the area, ΔC is the concentration difference between the two sides, and k is the relative permeability constant. For the example shown in Fig. 3, k is 1.50×10^{-8} $cm^2 sec^{-1}$, while for other samples k varied between 0.5 and 3 $\times 10^{-8} cm^2 sec^{-1}$.

The relative impermeability of plant cuticles to protons is not restricted to the sunflower. Epidermal strips from dark-grown soybean hypocotyls, taken from material identical to that used in our proton excretion studies (Cleland and Rayle, 1978), are only slightly permeable to protons unless the cuticle is abraded, whereupon the permeability is greatly enhanced (Table 1). Growing soybeans in the light does not cause a significant alteration in the proton permeability; unscrubbed strips are relatively impermeable while scrubbed strips have enhanced permeability (Fig. 4). Isolated leaf cuticles, obtained from the upper epidermis of Berberis sp. leaves, showed a permeability to protons similar to that of unscrubbed Helianthus hypocotyls. For example, the proton permeability of the leaf cuticle shown in Fig. 5 was calculated to be 0.9×10^{-8} $cm^2 sec^{-1}$. Tomato fruit cuticle is considerably less permeable to protons; in the example shown in Fig. 5 the rate of proton diffusion was lower in tomato fruit cuticle than it was with the leaf cuticle, despite a greater pH gradient across the cuticle.

Epidermal strips contain, in addition to the cuticle and its adhering cell walls, considerable protein, presumedly liberated from the frozen-thawed cells. This protein does not contribute to the impermeability of the strips to protons, as indicated by the fact that pretreatment of the strips with the proteolytic enzyme Pronase does not enhance proton permeability (Fig. 6). Partial removal of cuticular waxes with a chloroform-ethanol solution, however, markedly increases the proton permeability (Fig. 6).

The permeability to protons does depend upon the acid which is present. At pH 3 the proton permeability of unscrubbed sunflower epidermal strips is much greater when the acid is acetic acid, compared with HCl, phosphoric or citric acid at the same pH (Fig. 7). Apparently the cuticle is not permeable to any of the forms of HCl, phosphoric acid or citric acid which exist at pH 3, with the result that only free protons can cross the cuticle. On the other hand, the cuticle must be sufficiently permeable to the non-dissociated acetic acid molecule so that additional protons can be transported across the cuticle as part of this molecule.

Scanning electron microscopy has been used to assess the effect of scrubbing on the cuticle of sunflower hypocotyls. Unscrubbed strips show the expected smooth appearance of the cuticular surface (Fig. 8A). Occasional stomates are visible; whether they are functional or not is now known. We have observed no longitudinal cracks in the cuticle such so those seen by Hofer et al (1977) in auxin-treated wheat coleoptiles.

The surface of scrubbed strips has been markedly altered (Fig. 8B). Many small holes in the cuticle are visible, and often the cuticle shows large tears. It is apparent that scrubbing is an effective way to alter the integrity of the cuticle.

Discussion

There has been considerable uncertainty concerning the permeability of plant cuticles to protons. The ability of protons in the external medium to induce rapid elongation of coleoptile (Rayle and Cleland, 1970; Evans et al., 1971) and stem sections (Yamagata et al., 1974; Cleland and Rayle, 1975) suggests that some protons do penetrate the cuticle, although the growth-promoting protons could simply be entering the cut ends. The inability to detect significant auxin-induced acidification of the external medium with some dicot stem sections (Penny et al., 1975; Vanderhoef et al., 1977) has been taken by some to indicate that the cuticle is impermeable to protons (e.g., Rayle and Cleland, 1977) and by others to indicate that auxin-induced H+-excretion does not occur (e.g. Vanderhoef et al., 1977).

We have shown here that the cuticles of a leaf, of tomato fruit, and of growing soybean and sunflower stems are all relatively impermeable to protons, but that some protons will diffuse across the cuticle given sufficient time and concentration gradient. We cannot calculate a true diffusion constant for protons because the path length of diffusion is not known. The thickness of the cuticle could be estimted, but

this value is not necessarily identical to the length of the diffusion barrier (Norris, 1974; Schönherr, 1976). It is possible to calculate a relative permeability constant which disregards the length of the diffusion path. The value for protons, which we calculate to be 0.5-3 x 10^{-8} cm^2 sec^{-1} for both leaf and hypocotyl cuticles is similar in magnitude to that obtained for K^+ diffusing across apricot leaf cuticle (McFarlane and Berry, 1974). Because protons have a smaller atomic radius than K^+, one would expect the cuticle to be more permeable to protons, but until K^+ and H^+ permeability constants are determined on the same cuticle it will not be possible to confirm this. The permeability of apricot leaf cuticle to K^+ is markedly reduced by low pH, apparently due to production of positive charges in the cuticular pores which reduce the pore diameter (McFarlane and Berry, 1974). This suggests that proton excretion within the tissue will cause the permeability of the cuticle to protons to decrease even further. We have also shown here that scarification of the cuticle greatly increases the ability of protons to enter the tissue.

Can the relative impermeability of these cuticles to protons be explained as an artifact of the preparation of the experimental material? It is known that the permeability of cuticles can be altered by some isolation procedures, especially chemical ones (Norris and Bukovac, 1969), but the effect is an increase rather than a decrease in the permeability. Here the cuticles from the stem tissues were isolated by mechanical means, which might be expected to produce cracks or holes in the cuticle, but apparently this occurs only rarely. Scarification with emory, on the other hand, does lead to the appearance of large holes in the cuticle, which explains the enhanced permeability of these cuticles to protons.

One fact suggests that the proton permeability of the cuticle *in vivo* may be greater than that which we have measured. A pH 3 solution will cause live sections to elongate after a lag of only 2-5 minutes (Rayle and Cleland, 1973). Our measurements of the proton permeability would suggest that few protons would have crossed the cuticle in this time period. Elongation may simply be promoted by protons which diffuse in through the cut surfaces, but alternatively, the permeability of the cuticle may be greater *in vivo* that it is *in vitro*. For one thing the stomates may be open *in vivo*, providing some conduits for proton movement. Another possibility is that the permeability may be greater in cuticles which are elastically extended, as they will be in turgid, live material, as compared with the contracted, frozen-thawed material. In any case, while the permeability *in vivo* may be slightly greater than it is *in vitro*, it cannot begin to approach the permeability of scrubbed sections.

The relative impermeability of the cuticle to protons is consistent with the fact that much higher external proton concentrations are needed to induce the same growth rate in sections with intact cuticles as compared with scrubbed or peeled sections (Rayle, 1973). Protons excreted by cells in response to auxin will similarly be trapped within the tissue unless the cuticle is removed or abraded; the inability to measure auxin-induced proton excretion from sections with intact cuticles is consequently to be expected. The permeability of the cuticle to protons is enhanced by the presence of a weak monovalent acid such as acetic acid. We would predict that greater growth of intact sections would be induced by a pH 4

acetic acid solution, as compared with a citric acid or HCl solution.

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Table 1.

Proton movement across epidermal strips. Epidermal strips were peeled from light-grown sunflower hypocotyls or dark-grown soybean hypocotyls, frozen-thawed, and mounted in apparatus shown in Fig. 1. 5 ml of 10 mM KCl, pH 3.0, was placed against the outside surface and 100 ul of 10 mM KCl, pH 6.5 was placed against the inner surface. The pH was monitored continuously.

	Inside pH at 10 min.	
Material	Unscrubbed	Scrubbed
Dialysis tubing	3.85 ± 0.09	--------
Sunflower hypocotyl	5.55 ± 0.13	3.58 ± 0.13
Soybean hypocotyl	6.12 ± 0.15	3.83 ± 0.05

FIGURE LEGENDS

Fig. 1. Apparatus for measuring proton diffusion across cuticles. Epidermal strip or isolated cuticle (B) is glued across a 1x6 mm slit in a 5 ml plastic beaker cup (A). A 100 ul drop of 10 mM KCl, pH 6.5 (C) is placed on the inner side and a combination pH electrode (D) is inserted into the drop. The beaker cup is lowered into a 15 ml beaker containing 10 mM KCl, pH 3.0 (E), and the pH in C is continuously recorded.

Fig. 2. Movement of protons across scrubbed and unscrubbed epidermal strips of light-grown sunflower hypocotyls. Pieces of unscrubbed (intact), scrubbed sunflower hypocotyl epidermis or dialysis tubing were tested for proton permeability, using the apparatus in Fig. 1. Insert shows the curve for the dialysis tubing plotted as $-\log((H^+)_o-(H^+)_i)$ vs. time and shows the straight line expected for a diffusion process.

Fig. 3. Movement of protons across unscrubbed sunflower epidermal strip over long time periods. Epidermal strip was treated with Pronase prior to use. Insert shows the same curve plotted as $-\log((H^+)_o-(H^+)_i)$ vs. time, and shows the long lag before a linear diffusion curve is obtained.

Fig. 4. Comparison of proton permeability between scrubbed and unscrubbed (intact) light-grown soybean hypocotyl epidermal strips. Conditions same as in Fig. 1.

Fig. 5. Proton permeability of enzymatically-isolated cuticles from tomato fruit and Berberis sp. leaves. Condition are the same as in Fig. 1 except that the external (lower) solution was pH 3.0 for the fruit and 3.8 for the leaf cuticle. Insert shows the curve for the leaf cuticle replotted as $-\log((H^+)_o-(H^+)_i)$ vs. time in min.

Fig. 6. Effect of removal of proteins and cuticular waxes on proton permeability of sunflower hypocotyl epidermal strips. Strips were treated for 2 hr. with 0.2 mg/ml Pronase, or the hypocotyls were wiped twice with a 3:1 chloroform:ethanol mixture before peeling off the epidermal strip. Conditions are the same as in Fig. 1.

Fig. 7. Effect of acid on proton permeability of sunflower epidermal strips. Conditions were the same in each case except for the acid used to titrate the outside KCl solution to 3.0. Acids used were 0.01 M acetic acid, phosphoric acid and citric acid. The curve for HCl is similar to that for phosphate or citrate.

Fig. 8. Effect of scrubbing on the cuticular surface of the sunflower hypocotyl as viewed in the scanning electron microscope. a) Intact, unscrubbed. 750X. b) scrubbed, 750X. Note the disruption of the cuticular surface caused by the scrubbing.

Fig 1

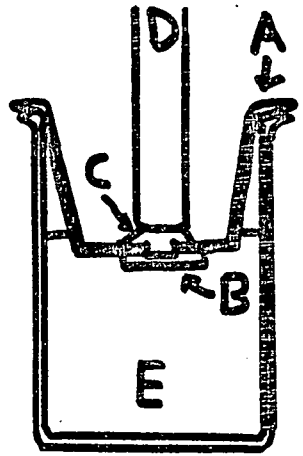
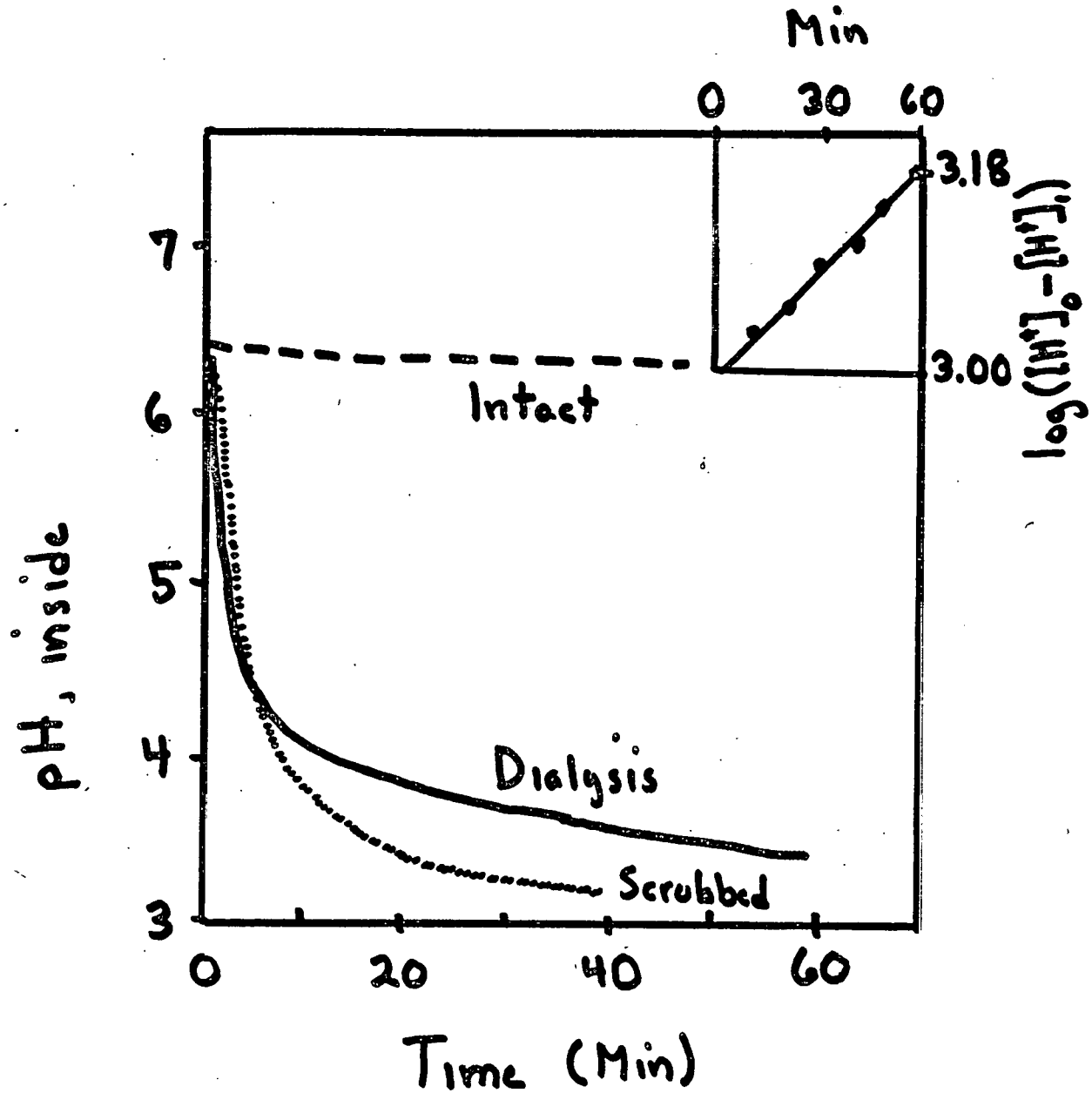


Fig 2


Min
0
30
60
3.18
3.00
$\log([H]-[H]_0)$
Intact
Dialysis
Scrubbed
pH, inside
7
6
5
4
3
0
20
40
60
Time (Min)

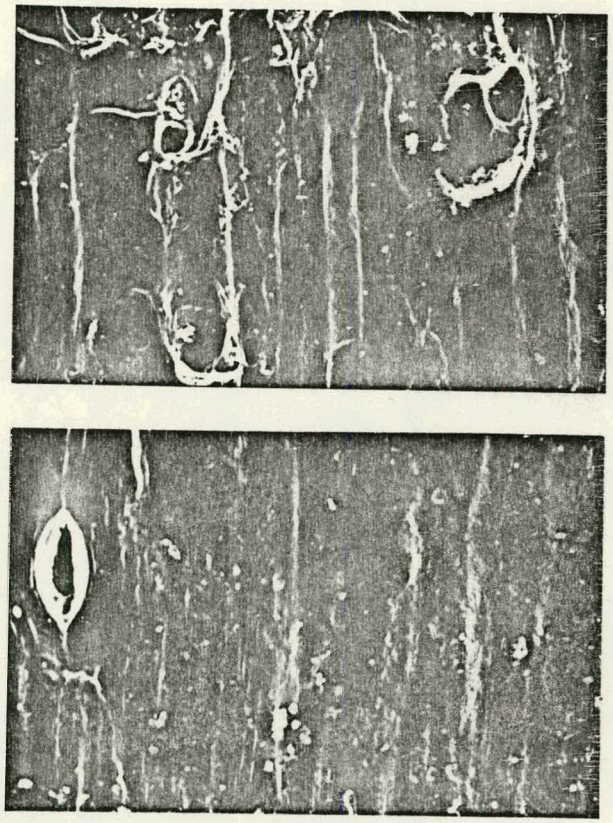


Fig 4

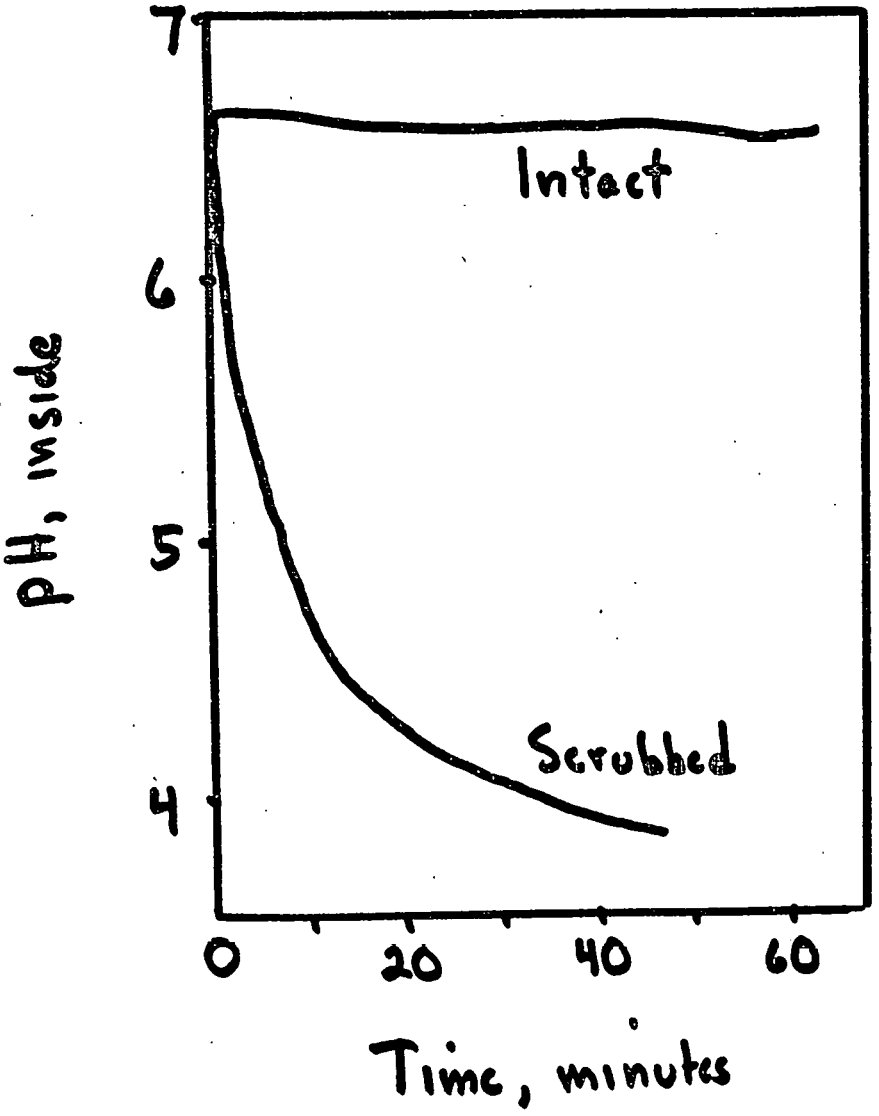


Fig 5

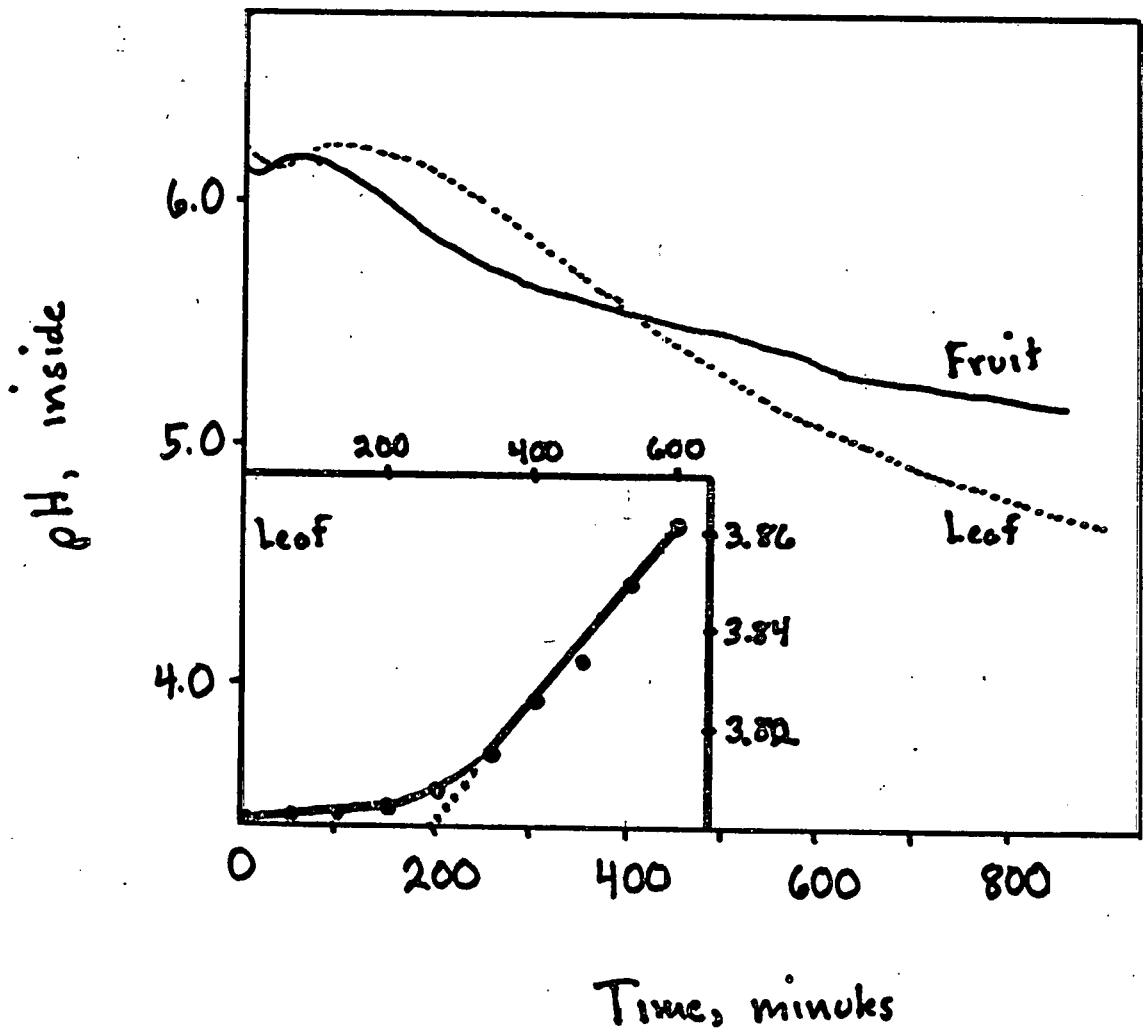

pH, inside
6.0
5.0
4.0
Fruit
Leaf
200
400
600
Leaf
3.86
3.84
3.82
0
200
400
600
800
Time, minuks

Fig 6

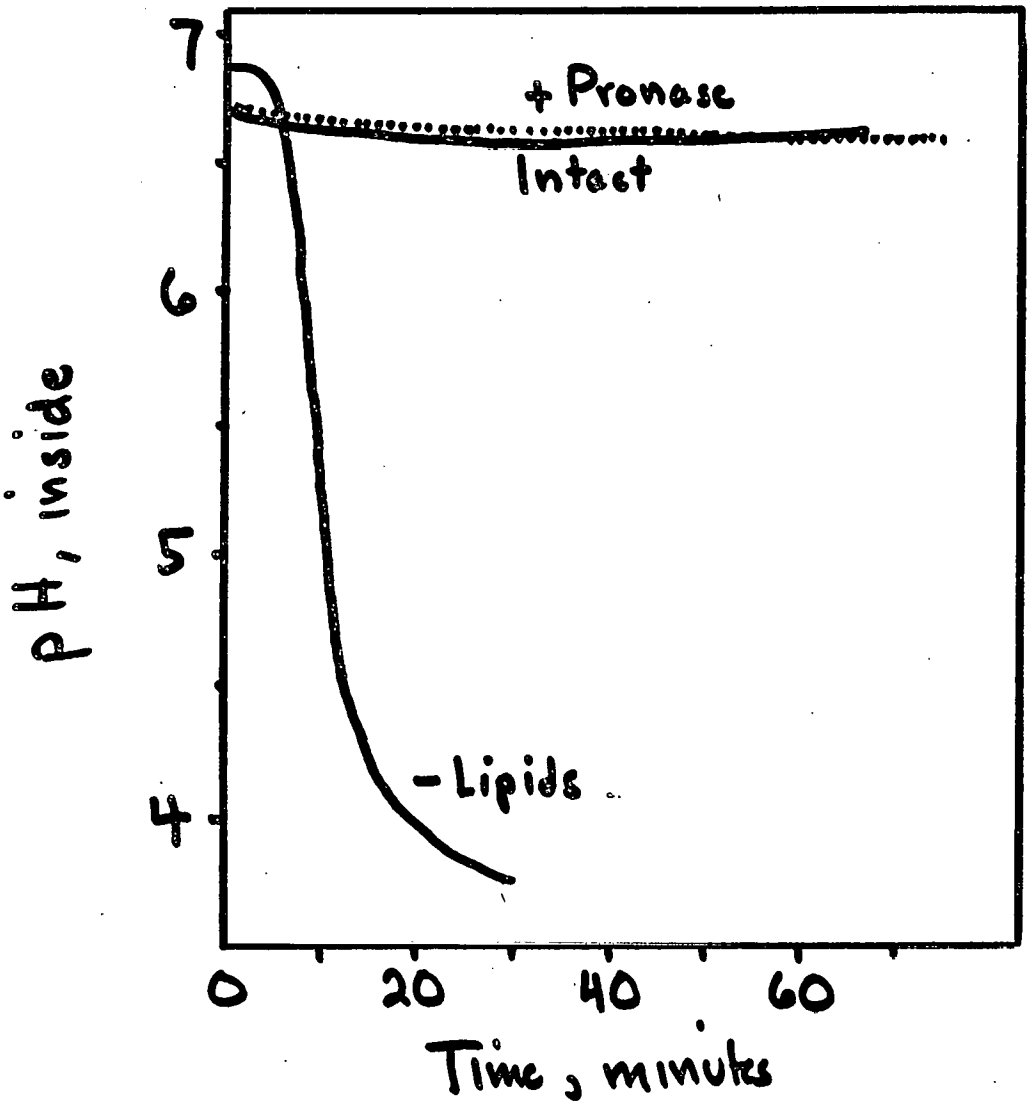
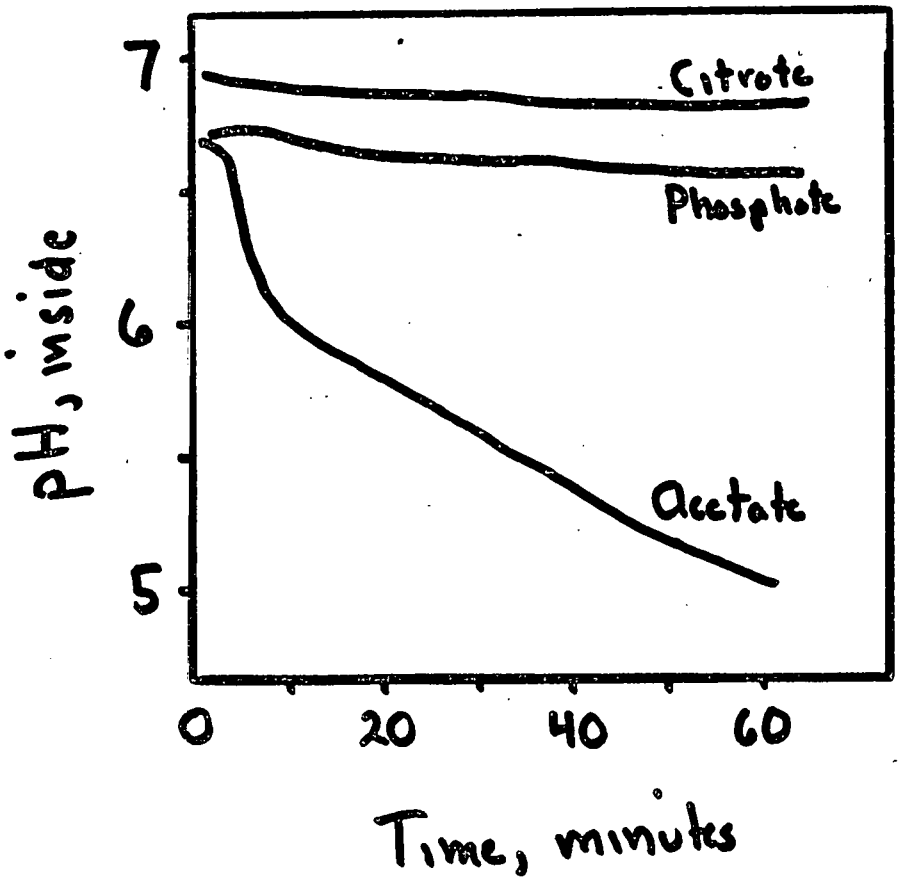


Fig 7


7
6
5
pH, inside
Citrate
Phosphate
Acetate
0
20
40
60
Time, minutes