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I am submitting herewith a dissertation written by Henry-York Steiner II entitled "The Effect of pH on Cell Wall Composition and its Relationship to Cell Aggregation in Carrot Cell Cultures." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

Donald Dougall April 29, 1993

Dr. Donald K. Dougall, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor and
Dean of the Graduate School

THE EFFECT OF pH ON CELL WALL COMPOSITION AND ITS RELATIONSHIP TO CELL AGGREGATION IN CARROT CELL CULTURES

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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ABSTRACT

Plant cell suspension cultures were used to investigate the effects of medium pH on cell aggregation in wild carrot cells. Two hypotheses were examined: 1) the origin of aggregates or the lack thereof in cell culture is a result of pH controlled, differential growth of aggregating or disaggregating genotypes, and 2) aggregate formation or dissociation is the result of the direct effect of H^+ ions on one or more components of the cell wall. Changes in aggregate size distribution of clones and cultures composed of a single size aggregate demonstrated that selection of an aggregating or disaggregating genotype was not involved in the formation of aggregates in culture. Cell walls were then analyzed for differences between the two phenotypes. Electron microscopy revealed less middle lamella in disaggregated cells grown at pH 4.5 and three times less wall bound Ca^{2+} than cells grown at pH 5.5. However, removal of Ca^{2+} from the tissue grown at pH 5.5 did not cause disaggregation nor did excess Ca^{2+} in cultures grown at pH 4.5 cause aggregation. It was concluded that changing the cell wall Ca^{2+} content was not sufficient to produce the changes in carrot cell aggregation. Cell wall fractionation showed the carbohydrate composition between the two phenotypes to be very similar while the total amount of cell wall material per unit volume of culture was 26% higher in the aggregates grown at pH 5.5. The amount of uronic acid was significantly higher in high molecular weight polymeric wall material released into the media of

disaggregated cultures. All fractions of cell wall from aggregated tissue grown at pH 5.5 had significantly greater amounts of hydroxyproline than did fractions from non-aggregated tissue grown at pH 4.5. In addition, the β-galactosidase activity was lower in non-aggregated tissue suggesting that cell wall degradation by this enzyme is not involved in the aggregation/disaggregation of cells seen in this system.

The results support a hypothesis that the effect of pH on carrot cell aggregation does not involve large changes in the existing cell wall structure alone, but arises from a pH controlled inhibition or activation of synthesis or incorporation of wall material into the existing wall. Prolonged growth at either pH 4.5 or pH 5.5 results in structural weakening or strengthening respectively and leads to the disaggregation or aggregation of the carrot cells in culture.

TABLE OF CONTENTS

Sections

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Dimethyl sulfoxide ..... DMSO

Phenol/acetic acid/water ..... PAW

Trans-1,2-diaminocyclohexane-N,N,N',N',-tetraacetic acid ..... CDTA

Ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid ..... EGTA

2-(N-morpholino) ethanesulfonic acid ..... MES

Cell wall material ..... CWM

Hydroxyproline containing glycoprotein ..... HPCG

Dry Weight ..... DW

Fresh Weight ..... FW

Degree of Methylation ..... DOM

Coefficient of Variation CV

Aggregate Size Distribution ..... ASD

Standard Deviation ..... SD

1. INTRODUCTION

The growth of plant cells in culture is known to be influenced by the pH of the medium in which the cells are grown (Dougall 1980). Changes in pH are also known to initiate and control a number of important metabolic processes in the plant cell (Smith and Raven 1979, Felle 1988). For example, experiments in our lab indicate that culture pH influences the morphology of carrot cells in suspension culture which ultimately affects the rate of ammonium uptake (Steiner 1987). The change in morphology involves aggregation or disaggregation of the cells at pH 5.5 or 4.5 respectively. The formation of aggregates has been identified as a first step in morphological differentiation as well as a serious obstacle in the exploitation of plant cell cultures for commercial purposes (Street et al. 1970, Shuler 1986, Staba 1985) and therefore a significant phenomena from both a basic and biotechnological research standpoint.

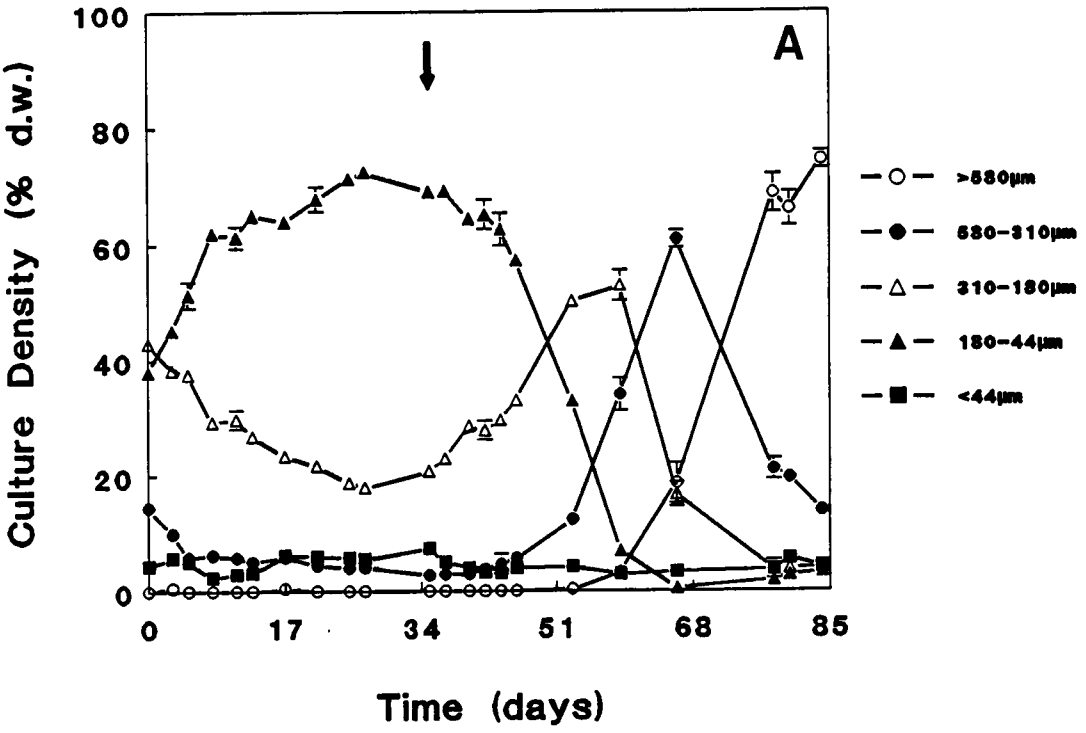
How pH causes plant cells to aggregate or disaggregate is unclear. The action of hydrogen ions may affect growth of differentially aggregating genotypes or may involve specific changes in the cell wall either by acting directly on one or more of its components or by affecting the synthesis of the wall polymers. Thus, the purpose of this study is to investigate the primary affect of pH with respect to cell aggregation and to characterize the initial biochemical events of aggregate formation and separation in plant cell cultures.

Medium pH and Aggregate Size Distribution in Carrot Cell Cultures

During the course of growing carrot cells semi-continuously, the cultures become aggregated or disaggregated depending on the pH of the medium. At pH 5.5 or greater the culture becomes highly aggregated. At pH 4.5 the culture is a fine suspension. If the pH of the two cultures is shifted in the opposite direction (i.e. pH 4.5 to pH 5.5 and vice versa), the aggregation of the cells shifts accordingly (figure 1). In pH 5.5 cultures, more than 60% of the cells are in aggregates 580 μm in diameter or larger and aggregation as high as 90% has been achieved in other experiments. Following a switch in the medium pH to 4.5 at day 35, the culture subsequently becomes composed of 180-44 μm aggregates while the >580 μm size class becomes less than 5% within 15 days. The reverse of this change also occurs. Seventy-five percent of cultures grown at pH 4.5 for 34 days are composed of 180-44 μm size aggregates. When these cultures are transferred to media whose pH is 5.5, 75% of the culture is composed of aggregates greater than 580 μm, and aggregates 180-44 μm in size comprise less than 5% of the culture by day 80 (Figure 1B). This shift in aggregation state requires 40 days to accomplish and progresses through a series of maxima beginning with the smaller size class where 310-180 μm clumps become maximal at day 55, 580-310 μm clumps become maximal at day 68, and clumps greater than 580 μm become maximal at day 80.

Two explanations are possible to account for the changes in aggregate size distribution seen in Figure 1. The RWC-63 cell line, which serves as inoculum for

Figure 1. The Effect of Medium pH on the Aggregate Size Distribution in Carrot Cell Cultures. 100 ml cultures were inoculated with 5 ml of 7 day old RWC-63 at pH 4.5 (A) and pH 5.5 (B). Cultures were grown semi-continuously for 34 days and the size class distribution of each culture was determined from 20 ml samples taken at daily intervals (Steiner 1987). On day 35 (arrows), the tissue was suspended in medium of opposite pH (i.e. 4.5-->5.5 and vice versa), grown semi-continuously and samples for size class distribution taken for another 49 days. Values are mean±SD of three replicate flasks at each pH. The absence of error bars indicates that the SD is smaller than the size of the symbol.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80
60
40
20
0
0
17
34
51
68
85
Time (days)
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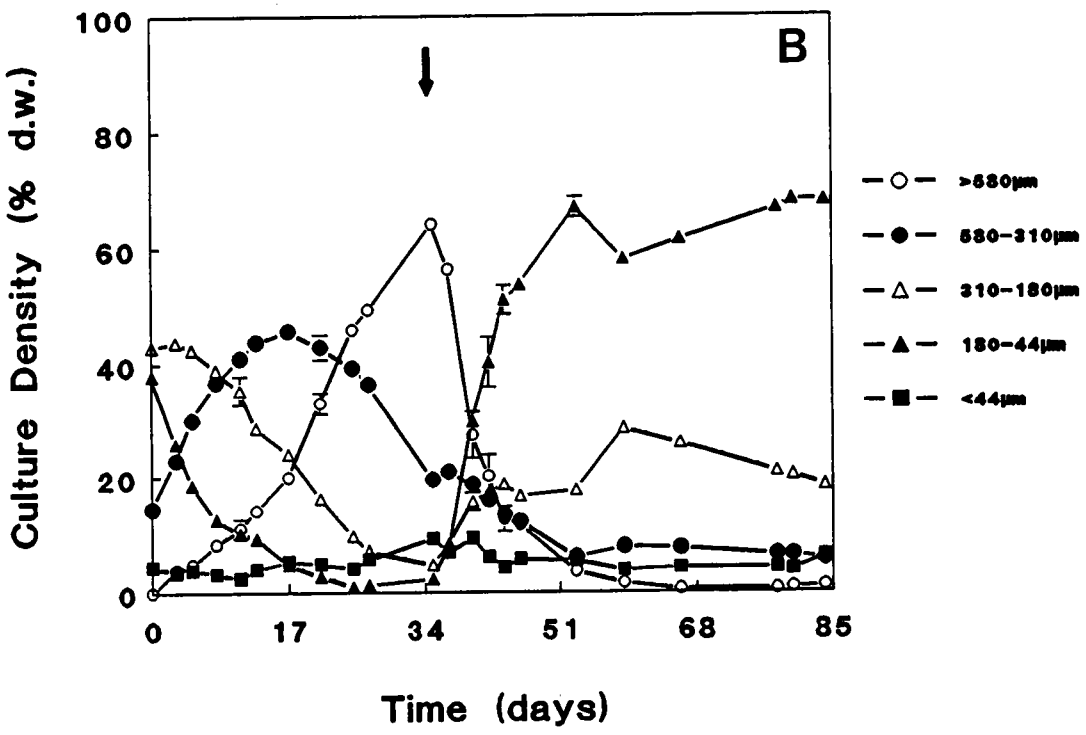
180–44µm
<44µm


B
Culture Density (% d.w.)
100
80
60
40
20
0
0
17
34
51
68
85
Time (days)
>530µm
530–310µm
310–180µm
180–44µm
<44µm

these cultures, contains a roughly equal distribution of aggregate sizes. Since the cell line was derived from petiole tissue composed of various cell types, it is possible that the different size aggregates present in the culture could reflect different cell genotypes. If the rate of growth of these individual aggregating genotypes was dependent on the pH of the medium, it is possible that the changes in aggregate size distribution could be accomplished by pH dependent differences in growth rates of aggregating and non-aggregating genotypes within the same culture. For instance, medium at pH 4.5 would favor growth of a non-aggregating cell type while decreasing the growth rate of an aggregating cell type. Medium at pH 5.5 would produce opposite results. This differential effect of pH on growth rate could explain the distribution of aggregate sizes prior to changing the medium pH (day 35) seen in Figure 1A and 1B. The presence of the smaller percentages of aggregates would indicate other cell types which express other, various states of aggregation. When cells grown at pH 4.5 are incubated at pH 5.5 (day 35 and on, Figure 1A), the growth rate of the non-aggregating cell type would decrease and its decline from the culture would be facilitated by the daily, 20 ml removal of culture during semi-continuous culture. Concurrently, the aggregating genotype present in small amounts would now experience an increase in growth rate relative to that of the non-aggregating cell type and begin to sequentially move up through the higher size classes as depicted for days 35-84 in Figure 1A. The growth rate of the non-aggregating cell type in this culture would have to be reduced to a level such that a small population of these cells would always be present, thus maintaining the reversibility of the aggregate size distribution.

Physiologically, this explanation of the effect of pH on aggregate size distribution implies that the genotypes express one aggregate size only and that pH effects the rate of cell division within the aggregate and not the composition or synthesis of the cell wall. To account for the different sizes of aggregates, the cell types would inherently produce walls of different strengths and the size of the aggregate, characteristic of that genotype, would be determined by other factors (such as shear rate), but pH would not influence the strength or weakness of the wall.

If pH does not differentially effect growth rates, then direct effects of pH on cell wall components is an alternative explanation. Three possible mechanisms are apparent: 1) pH effects wall components already present in the wall resulting in loss of the component from the wall (degradation), 2) pH effects the synthesis and/or the incorporation of wall components into the wall, or 3) pH induces structural changes in the wall components without loss of the structural member. pH induced loss of wall polymer could result from cleavage of acid labile linkages between polymers, destabilization of pectin by H^+ ion exchange with Ca^{2+}, degradation of polymers by induction of hydrolytic enzymes, or inhibition of phenolic cross-links between polymers or structural glycoproteins or a combination thereof. The different pHs could also inhibit or decrease the activity of enzymes or enzyme complexes involved with the synthesis of polysaccharides, glycoproteins, or phenolic residues. The effect of pH may also cause structural rearrangements within a polymer or between polymers or cause a redistribution of polysaccharide within the wall.

If the process of aggregate formation or separation is localized to the cell wall, the mechanism of cell separation presumably involves the shear forces generated in the rotating culture flask acting on the aggregates. Shear stress is directly proportional to shear rate which, in the case of the carrot cultures, is proportional to the rotation rate of the culture flasks (Aiba et al. 1973). Therefore, the rotation rate combined with the strength or cohesiveness of the cell wall would determine the aggregate size distribution of the culture. At constant agitation rate, the greater the cohesive force between the cell walls the greater the possibility of aggregate formation or maintenance of aggregates. Likewise, the weaker the association, the smaller and more numerous the aggregates. This pH effect on wall integrity, coupled with the shear forces could explain the aggregate size distributions seen in Figure 1.

The above information can be condensed into two general hypotheses: 1.) **Selection Hypothesis.** The population used to initiate the cultures at pH 4.5 and 5.5 contains a mixture of both aggregated and dispersed cells, comprised of two different cell genotypes one of which grows as an aggregate, the other grows as a dispersed suspension. Further, in such a mixed population, pH 4.5 allows the dispersed cell type to grow at a faster rate than the aggregated cell genotype and correspondingly, media at pH 5.5 allows the aggregated cell genotype to grow at a faster rate than the dispersed cell genotype thus producing the pH dependent aggregation.

2.) Wall Composition Hypothesis. The pH at which carrot cells are grown affects the rate of degradation, rate of synthesis, or structure of wall components resulting in strengthening or weakening of the wall. Shear forces generated by constant agitation

then produce a change in the aggregate size distribution depending on the medium pH. Thus at pH 4.5, the strength of the wall is reduced relative to that of pH 5.5 and leads to fragmentation of aggregates during shaking and conversely, the wall is stronger at pH 5.5 and resists dissociation by shear forces.

Information available in the literature does not allow one to determine which of the two hypotheses is operational in the carrot system. Very little has been published which directly concerns the effect of pH on formation and separation of plant cells in culture. No information is available on the effect of pH on growth rates of different cell types in the same culture so the Selection Hypothesis must be tested directly to assess its role in aggregation. By far, the majority of the literature is related to elements of the Cell Wall Hypothesis. The remainder of the discussion will examine some of the previous findings and their connection to the effect of pH on the plant cell wall.

Formation and Separation of Aggregates in Plant Cell Cultures.

The formation of aggregates in plant suspension cultures has been previously observed (Street et al. 1965, Halperin 1971, Halperin and Minocha 1973, Wallner and Nevins 1973, Wallner and Nevins 1974, Kuboi and Yamada 1978, Kinnersley and Dougall 1980). In four studies, plant growth regulators were shown to influence the aggregation state of cell cultures. 2,4-D both enhanced dissociation in tobacco suspension cultures (Kuboi and Yamada 1978) and inhibited cell separation in suspensions of Paul's Scarlet rose (Wallner and Nevins 1973). Cytokinins were shown

to retard cell separation (Halperin 1971), and benzyladenine promoted the activity of peroxidase which has been associated with lignification of the cell wall (Halperin and Minocha 1973, Pedreno et al. 1989).

Although there appears to be a relationship between growth regulators and aggregation, the action of these compounds on wall components could be more indirect (for example, stimulation or suppression of H^+ secretion by auxin), rather than direct. As of yet, there are no reports of a plant growth regulator directly affecting a component of the plant cell wall.

The only study that specifically identifies a mechanism for disaggregation of plant cells in culture involves the analysis of wall enzyme activity during growth of Paul's Scarlet Rose cells (Wallner and Nevins 1974). It was shown that activities of wall localized β-glucosidase and β-galactosidase increased in parallel as cellular aggregates began to fragment during the stationary phase of growth. The parallel increase in activities coincided with a sharp decrease in the galactose content of the cell wall polysaccharides which led Wallner and Nevins to postulate that the activities were that of one protein, a galactanase. Their results indicated that enzymatic degradation of wall constituents may have been involved in cell separation of rose cultures but did not reveal how this activity was induced or why its induction was coincident with the stationary phase. A β-galactosidase, with a pH optima of 3.7 has also been shown to degrade to a limited extent, wall material isolated from sycamore cells (Keegstra and Albersheim 1970). Together, these data lead to the idea that carrot cells grown at pH 4.5 might have higher β-galactosidase activity than cells

grown at pH 5.5 and this higher degradative activity would weaken the wall enough to cause cell separation analogous to what was observed in the rose cultures.

Thus, of the few reports which directly concern plant cell aggregation, only one specifies a possible mechanism which may account for the development of aggregates in culture. However, it is possible that the effect of pH on other wall elements may precede the activity of hydrolytic enzymes or be necessary in tandem with the enzymes to effectively loosen the wall and produce the characteristic aggregate development. It is therefore necessary to look at other factors which effect the integrity of the cell wall and how they might be related to or influenced by pH.

The Relationship Between pH and Factors Which Influence Cell Wall Composition.

Events connected with wall extension and wall loosening, which are thought to involve changes in the wall composition, have been correlated with cell separation (Lamport 1964, Henshaw et al. 1966). Also, the reversible characteristic of the pH effect on carrot cell aggregation is similar to the reversibility of acid-induced wall loosening shown in cucumber hypocotyl sections alternately incubated in pH 7.0 and 4.5 buffers (Cleland et al. 1987). This reversible acid extension has also been demonstrated in oat coleoptiles (Tepfer and Cleland 1979), bean leaf (Van Volkenburgh et al. 1985), soybean hypocotyls and pea epicotyl tissues (Cleland et al. 1987) and suggests that pH induced aggregation and separation of carrot cells may have something in common with the postulated mechanisms of acid-induced wall loosening.

Secretion of H^+ ions, as a result of the application of auxin, is necessary for wall loosening to occur (Hager et. al. 1971, Rayle and Cleland 1977, Van Volkenburgh and Cleland 1980). H^+ ions are rapidly secreted upon application of auxin and exogenous H^+ ions mimic the wall loosening action of auxins in both *in vivo* and in isolated cell walls *in vitro*. Application of neutral buffers which destroy the acidification effect inhibit the action of auxins on wall loosening. In addition, H^+ ions may loosen walls of monocots (specifically grasses) indirectly by activating an enzyme which cleaves β-(1-->3)-glucan, a major component of the cell walls of cereals. However, this may not be the case for dicots (Cosgrove and Knievel 1987). The involvement of H^+ ions in wall loosening is well established and it is possible that a similar mechanism is involved in the aggregation/disaggregation of carrot cells. Thus far, the molecular target for H^+ ions in the cell wall has yet to be identified.

Calcium has also been reported to be a significant regulator of cell wall extensibility (Sarbjit and Cleland 1988, Jarvis 1982, Jarvis 1984, Baydoun and Brett 1984, Thom et al. 1982). Specifically, Ca^{2+} is involved in linkages between pectic substances in the cell wall. These linkages consist of Ca^{2+} bridges which are fixed by electrostatic interactions with the carboxyl groups of galacturonic acids on adjacent molecules of pectin and by coordination linkages with hydroxyl groups of other polysaccharides (Wuytack and Gillet 1978, Demarty et al. 1984). Blocks of non-methylesterified galacturonic acid residues will bind Ca^{2+} molecules in an organized fashion forming strong cross-links in an arrangement known as the 'egg box' model (Rees and Richardson 1966, Rees 1972, Grant et. al. 1973, Jarvis 1984, Powell et al. 1982, Rees 1977).

The Ca^{2+} appears to compete with H^+ for exchange sites (carboxyl groups of galacturonic acid) of the cell wall (Demarty et al. 1984). At hydrogen ion concentrations which occur during wall extension, Ca^{2+} can be displaced (Baydoun and Brett 1984), while increasing the pH increases binding of Ca^{2+} (Jarvis 1984, Baydoun and Brett 1984). This H^+/Ca^{2+} ion exchange has been implicated in wall softening during fruit ripening (Knee 1982) and a number of studies have suggested that the load bearing bonds which decrease wall plastic deformation are Ca^{2+} bridges (Jarvis et. al. 1984, Prat et. al. 1984, Soll and Bottger 1988, Virk and Cleland 1988). Additionally, calcium has been shown to reduce elongation in some stems (Tepfer and Taylor 1981). Similar events could occur in our carrot cultures where an increasing H^+ ion concentration could displace Ca^{2+} ions bound in the cell wall, causing the walls to weaken and separate. As pH is increased, calcium would recomplex with the wall, strengthening it, preventing dissociation and resulting in aggregation.

Other reports suggest that proteins play a vital role in acid-induced extension. Boiling in methanol (Rayle et al. 1970), digestion with pronase (Cleland and Rayle 1978), or treatment with 8 M urea (Tepfer and Cleland 1979) either inhibited or greatly reduced the wall extension response of tissue preparations when they were incubated in acidic buffers. The activities of cellulase (Hayashi et al. 1984) and β-galactosidase and β-glucosidase (Johnson et al. 1974) are enhanced by acid pH and have been implicated in acid-induced wall extension (Johnson et al. 1974, Yamamoto and Nevins 1981) and as mentioned before in cell disaggregation (Wallner and Nevins

1974). In contrast, peroxidase has been shown to cause dimerization of phenolic side-chains on polysaccharides and glycoproteins to form isodityrosine (Fry 1987), diferulate (Markwalder and Neukom 1976), and other wall strengthening cross-links. Peroxidase is inhibited by low pH (Rayle and Cleland 1977) and Ca^{2+} has been reported to cause changes in quantity and localization of peroxidase as well (Sticher et al. 1981, Penel and Greppin 1979). Conceivably, peroxidase would cross-link wall polymers at pH 5.5, strengthening the wall and increasing the ability of the cells to aggregate or resist disaggregation. Alternatively, at the lower pH, the decreased peroxidase activity would lead to the newly synthesized walls being weaker than the old ones and this result would become apparent in the aggregation state of the cultures only after the cells had grown and detached from the old cell walls.

Cellulase cleaves very little crystalline cellulose but does hydrolyse xyloglucan, the major hemicellulose of the primary wall (Hayashi and Maclachlan 1984). Existing wall cellulase is activated by low pH (below pH 5) which induces the sloughing and turnover of the xyloglucan (Nishitani and Masuda 1983, Hayashi et al. 1984). High cellulase activity has also been correlated with degradation of cell walls during abscission (Sexton and Roberts 1982) though it is not known whether pH is the primary stimulus of this activity.

The non-enzymatic glycoprotein extensin has also been hypothesized to have an integral role in cross-linking pectin molecules in the primary wall (Cassab and Varner 1988, Tierney and Varner 1987, Lamport 1986). Extensin, a highly glycosylated, rod shaped glycoprotein, is thought to be covalently bound in the cell

wall based upon the inability to extract the protein by methods which elute most other wall proteins. It has also been shown that extensin may be able to form its own cross-linked network independent of the structural polysaccharides in the wall. The cross-linking of extensin is thought to occur by the formation of isodityrosine from a tyrosine residue in each of two separate extensin molecules each of which are bound to the cell wall (Cooper and Varner 1984, Fry 1982, Epstein and Lamport 1984, Fry 1986). Everdeen (1988) suggested that this cross-linking event may be an enzymatic process involving an extensin peroxidase. In such a network, the formation of greater numbers of cross-links between extensin molecules or incorporation of more extensin in carrot cell walls at pH 5.5 would strengthen the wall and increase the production of aggregates. Reducing the number of cross-links between molecules or reducing the number of extensin molecules forming the network in the wall at pH 4.5 would weaken the carrot cell wall relative to that at pH 5.5 and increase the cell's ability to separate.

Thus, the effects of H^+ ions on wall enzyme activity may provide another mechanism by which wall polymers could strengthen or weaken the wall depending on how the enzyme affected the wall component and in what pH dependent direction.

Cell Wall Synthesis and Polymer Structure

Another mechanism to explain how pH might affect cell wall structure in connection with aggregation is synthesis of cell wall components. Matrix

polysaccharides (excluding callose and cellulose) and glycoproteins of the cell wall are synthesized mainly by the endo-membrane system involving the rough endoplasmic reticulum, golgi bodies, and golgi body-derived vesicles which transport the preformed polysaccharides and glycoproteins to the plasma membrane, fuse with the membrane and release and incorporate their contents into the cell wall (Northcote and Pickett-Heaps 1966, Fry 1988). Callose and cellulose are synthesized in the plasma membrane from nucleoside diphosphate sugars and incorporate into the wall directly (Delmer 1987, Wooding 1968). Regulation of cell wall synthesis is not completely understood but does involve supply or interconversion of nucleoside diphosphate sugars, activity of the polysaccharide synthases, and/or secretion and incorporation of the polysaccharides into the wall (Fry 1988). Fluctuating H^+ ion concentrations could increase or decrease any of these steps and lead to the phenomena seen in the carrot cell cultures. Changes in H^+ ion concentration affecting wall enzymatic activity or altering Ca^{2+} mediated vesicle fusion have been suggested as possible mechanisms effecting wall extension and synthesis respectively (Fry 1988, Ricard and Noat 1986).

Chemical changes in structural wall polymers have been identified as in the auxin induced nicking of xyloglucan which results in a decreased mean molecular weight of xyloglucan (Nishitani and Masuda 1983, Hayashi et al. 1984). It is not known whether pH is involved in the nicking. With very little information regarding changes in polymer structure, it is difficult to assess the possibility that this type of mechanism is involved in the development or separation of aggregates in carrot cell cultures.

Finally, two physiologically normal events which occur in a plant, abscission and fruit ripening, induce changes in the cell wall which may have some similarity with the processes occurring in the carrot cell cultures. Initially, pectin methylesterase was implicated in the process of wall breakdown during abscission but subsequent reports showed no consistent relationship between abscission and pectin methylesterase activity (Sexton and Roberts 1982). Current data show that at the onset of abscission, a cellulase unique to the abscission zone appears and is one of the first events in the abscission process (Sexton and Roberts 1982). Though it is unclear what cellulase attacks in the separation zone walls, the enzyme is partly responsible for wall loosening in this specialized area and could be a factor in cell separation. In fruit ripening, polygalacturonase has been shown to cause the polyuronide degradation characteristic of ripening fruit but does not cause the associated fruit softening (Giovannoni et al. 1989). It is unclear whether cellulase or polygalacturonase could be involved in cell separation or aggregation to the extent seen in the carrot cultures. Neither of these two processes have been investigated for a correlation of their respective enzyme activities with pH though it would be expected that pH would have some effect on the catalytic rates.

Summary

Carrot cells when grown in a buffered media at pH 4.5 become a fine suspension while carrot cells grown at pH 5.5 aggregate into large clumps. The causes for this differential response to medium pH are unknown. The events could

be located in the cell wall and be associated with either the formation of the cell wall during growth or with existing wall structure. In addition, in the mixed population of the culture, different genotypes may be expressing different growth rates in response to the different pH.

It is evident from the above discussion that a number of factors can produce changes in the structure or composition of the wall which could lead to the aggregation states seen in the carrot cultures. H^+ ions appear to be one of the first steps in acid-induced wall loosening but the target of the H^+ ions is unknown. Ca^{2+} can weaken or stiffen the wall polymer network and H^+ ions can alter the levels of Ca^{2+} in the wall. Wall proteins/enzymes have also been implicated in both aggregation and acid-induced wall loosening. A mechanism involving the cross-linking of structural proteins such as extensin through isodityrosine is also a candidate for involvement in acid-induced wall loosening (Lamport 1986). Changes in wall synthesis or in the structure of the wall polymers have also been discussed and are also possible mechanisms.

The large number of possible factors which could lead to the phenomena being investigated requires focusing on those which are closely associated with pH and wall structure. A number of enzymes have the ability to effect wall structure but only β-galactosidase has been directly connected with aggregation and thus was selected to be assayed. Based on these considerations and the information previously discussed, the following experimental strategy was adopted:

1. test the validity of the selection hypothesis

2. characterize cell wall ultrastructure
3. analyze cell walls for Ca^{2+} content
4. measure β-galactosidase activity in aggregates
5. determine composition of cell wall and extracellular material by carbohydrate, hydroxyproline, protein, degree of methylesterification, and molecular weight analyses.

What follows are results of these experiments and a discussion of their relationship to pH controlled formation and separation of aggregates in carrot cell suspension cultures.

2. MATERIALS AND METHODS

Plant Tissue.

Source material used throughout this study was the wild carrot (*Daucus carota*, L.) cell line RWC-63 isolated from a petiole section (Wetherell and Dougall, 1976). This tissue was maintained as a stock culture in 125 ml Erlenmeyer flasks containing 25 ml of WCM-1 medium (Wetherell, 1969). Cultures were maintained in the dark at 25°C, shaken on a rotary shaker at 120 rpm, and subcultured done 14 days at 1:20 density.

Culture Conditions.

Experimental cultures were grown by a semicontinuous culture methodology in WCM-4 medium (Dougall and Weyrauch, 1980), an improved media for anthocyanin production buffered with potassium succinate (20 mM). All media were additionally buffered with MES (30 mM) and media were filter sterilized using a Gelman 0.2 μm pleated cartridge. 95 ml of WCM-4 adjusted to pH 4.5, or 5.5 was added to 500 ml Erlenmeyer flasks equipped with silicon sponge enclosures. Flasks were inoculated with 7 day old WC-63 suspension at an initial density of 1:20. After a 3-4 day adaption period, daily cycles of media turnover began. 20 ml of each culture was removed, followed by the addition of 20 ml of fresh media. In addition,

1-2 ml of sterile water was added to each flask on a daily basis to compensate for evaporation. The amount of water added was established in preliminary experiments by weighing cultures on a daily basis, taking an average daily gram weight loss per day per culture and converting the number to milliliters of water lost per day per culture. Every week the cultures were transferred to new flasks to check culture volume and minimize cells adhering to the walls of the flask.

Selection and Growth of Clonal Cultures.

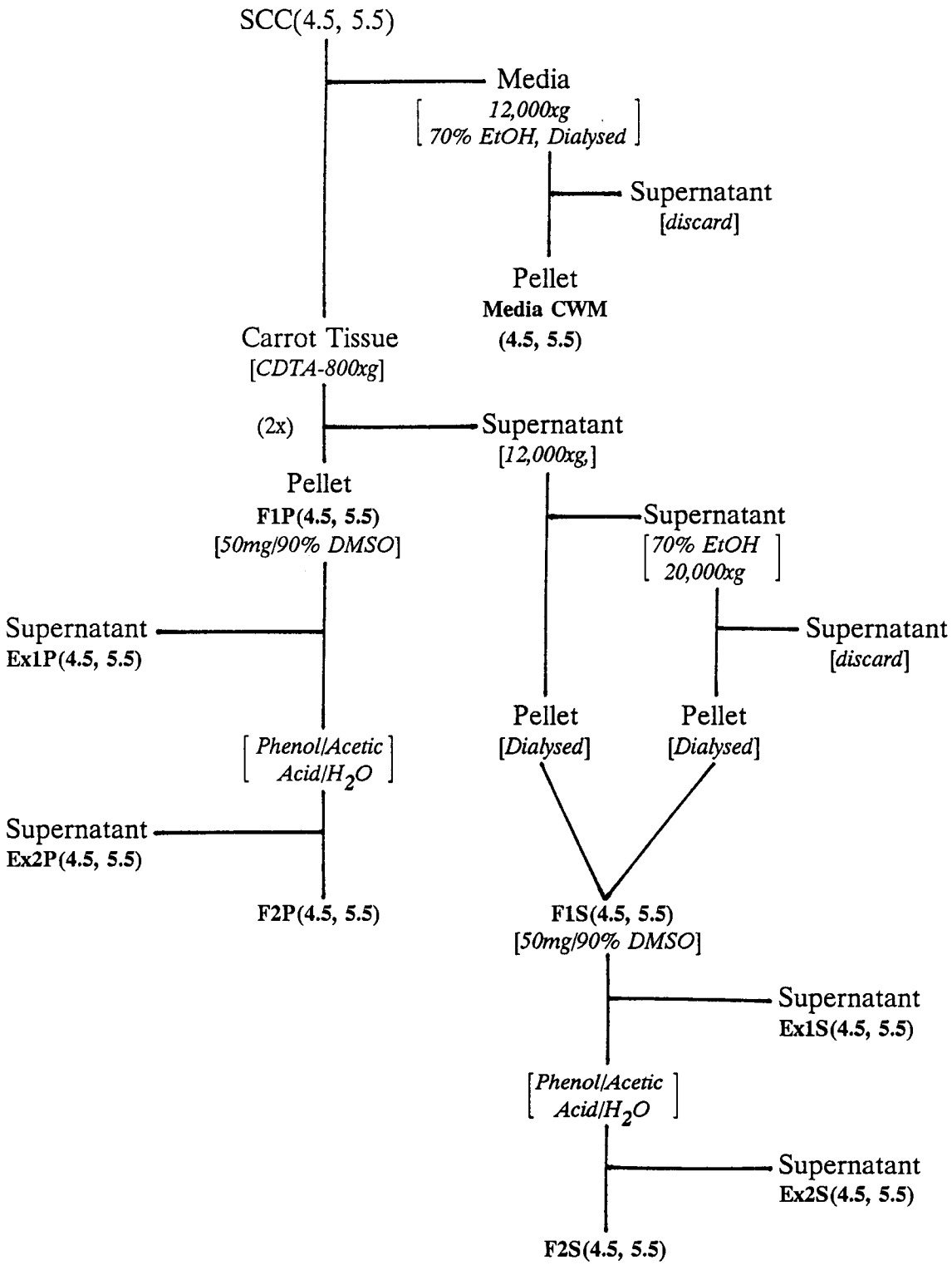
Clonal cultures were initiated according to Dougall et al. (1980). Inocula used was 7 day old RWC-63 stock culture screened through a 44 μm stainless steel screen and the filtrate collected. The tissue retained on the screen was washed several times with sterile WCM-1 media and the filtrate added to that which was collected from the screening. The combined filtrates were centrifuged at 200 x g for 5 min. and 90 % of the medium was removed and the pellet resuspended in the remaining media. The concentration of single cells and multi-cell aggregates was determined by counting in a hemocytometer. Aggregates were counted as single unit. The suspension was diluted with WCM-1 media to give 100 cells or aggregates per 1 ml and used as inoculum for plating. Plates were made with WCM-1 supplemented with 10 mM myo-inositol and 0.5% agar washed according to Binns and Meins (1979). The pH was adjusted to 5.8. One ml of inoculum was mixed with 10 ml of plating medium at 45°C and allowed to set in 100 mm petri dishes. Plates were wrapped in Para-film and kept at 25°C in the dark. When colonies were approximately 1 mm

in diameter, well isolated colonies were transferred by sterile wire loop to 16x150 mm screw cap tubes containing 2 ml of WCM-1. Tubes were rotated on a rotary drum shaker for 3 weeks after which 2 ml was transferred to 25x150 mm tubes containing 10 ml WCM-1 media. After 1 week of growth, 2.5 ml of this culture was used to inoculate replicate 125 ml Erylenmyer flasks containing 25 ml WCM-4 medium and grown semi-continuously at 20% dilution for 20 days.

Cell Wall Isolation.

Cell walls were isolated from three independent experiments, each consisting of three 100ml cultures (Figure 2). The entire contents of each culture was collected by vacuum filtration through 47 mm Whatman GF/A glass fiber filters. Tissue was washed with approximately 25 ml of water and the tissue from the three flasks was combined and weighed. 3-4 g FW of the combined tissue was suspended in 50 ml of CDTA buffer (50 mM sodium phosphate (dibasic), 50 mM CDTA, pH 6.8) and stirred sealed under nitrogen at 5°C for 24 hrs. CDTA treated material was centrifuged at 800 x g for 10 min. in a Beckman TJ-6 centrifuge. The supernatant was removed and the pellet reextracted with the CDTA buffer as above. This first supernatant was centrifuged at 12,200 x g for 20 min. in a Beckman J21C centrifuge. The resulting supernatant was precipitated with four volumes of ethanol at a final concentration of 70% (v/v) at 5°C for 24 hrs while the pellet was resuspended by sonication in 10 ml of water and stored at -20°C. The ethanol precipitated material was recovered by centrifugation at 20,000 x g for 20 min., resuspended by sonication

Figure 2. Cell Wall Isolation Scheme. The scheme begins with semi-continuous tissue either at pH 4.5 or 5.5 and ends with the isolated fractions. The fractions are indicated in **bold** and key procedures are indicated by bracketed [*italics*].


SCC(4.5, 5.5)
Media
12,000xg
70% EtOH, Dialysed
Supernatant
[discard]
Pellet
Media CWM
(4.5, 5.5)
Carrot Tissue
[CDTA-800xg]
(2x)
Supernatant
[12,000xg,]
Pellet
F1P(4.5, 5.5)
[50mg/90% DMSO]
Supernatant
[70% EtOH
20,000xg
Supernatant
[discard]
Supernatant
Ex1P(4.5, 5.5)
Pellet
[Dialysed]
Pellet
[Dialysed]
[Phenol/Acetic
Acid/H2O
Supernatant
Ex2P(4.5, 5.5)
F2P(4.5, 5.5)
F1S(4.5, 5.5)
[50mg/90% DMSO]
Supernatant
Ex1S(4.5, 5.5)
[Phenol/Acetic
Acid/H2O
Supernatant
Ex2S(4.5, 5.5)
F2S(4.5, 5.5)

in 2 ml of H_2O, and stored at -20°C. The second supernatant from the second CDTA extraction contained much less material and was precipitated directly at 70% (v/v) EtOH and the

insoluble material recovered by centrifugation at 20,000 x g for 20 min. and stored at -20°C. A third extraction with CDTA yielded no material in the supernatant. The final CDTA-extracted pellet was washed twice in 10 ml of H_2O and centrifuged at 1000 x g for 10 min. This pellet was resuspended in 10 ml of H_2O and stored at -20°C. To insure that all the calcium was removed by the CDTA treatments, portions of the discarded supernatants from each of the three CDTA extractions were analyzed for Ca^{2+} by atomic absorption spectrometry. 95% of the total Ca^{2+} extracted by the CDTA treatments was removed in the first treatment, 5% in the second, and none in the third. Small portions of the cell wall containing samples from each of the three treatments were also measured and showed a reciprocal drop in Ca^{2+} concentration.

All of the EtOH precipitated material, including the pellet from the first supernatant, was combined (designated F1S, pH 4.5 or 5.5; see Figure 2), dialyzed (Spectropore Standard Cellulose, 12-14K MW cutoff) against H_2O for 24hr. at 5°C with 4 changes of H_2O, and then freeze dried. The final CDTA extracted pellet (designated F1P, pH 4.5 or 5.5) was dialyzed and freeze dried in the same manner.

Media CWM was recovered with the addition of EtOH to the spent media filtered off of the collected tissue, to give a final concentration of 70%. After 24 hrs. at 5°C, the sample was centrifuged at 17,000 x g for 30 min. The supernatant was

discarded and the pellet resuspended in H_2O, dialyzed against water, and freeze dried.

Starch and Protein Extraction.

Starch was extracted from cell wall material by the method of Fry (1988) as follows. 50 mg of 4.5 F1S, 5.5 F1S, 4.5 F1P, and 5.5 F1P were each suspended in 5 ml of 90% DMSO and incubated for 6 hrs. with constant stirring at 25°C (Fry 1988). Samples were centrifuged at 12,200 x g for 25 min. The supernatant was recovered, and centrifuged at 20,000 x g for 30 min. to collect any CWM trapped in the viscous supernatant. Any CWM recovered was added to the pellet (prior to EtOH washings). The combined pellets were washed twice in 10 ml 70% EtOH and centrifuged as above to remove DMSO. Two volumes of H_2O were added to both the DMSO and ethanol supernatants followed by freeze drying. Residues from the DMSO and ethanol supernatants were combined and called the starch containing Ex1S or Ex1P fraction. Small portions (<0.5 mg) from the pellet and supernatant were tested for the presence of starch with 0.33% I_2 plus 0.67% KI (Fry 1988).

Protein was extracted from the DMSO-extracted residue according to Fry (1988) by suspending the residue in 5ml of PAW (phenol/acetic acid/H_2O; 66.6%, 26.6%, 6.8%, v/v) and stirred for 6 hrs at 25°C. Samples were centrifuged at 12,200x g for 25 min. The pellets were washed 3x with 70% EtOH, had twice the volume of H_2O added, freeze dried and labeled F2S or F2P. To recover any material dissolved in the PAW, all of the PAW supernatants from a single tissue sample were combined,

suspended in EtOH to give a final concentration of 70% EtOH, and incubated at -5°C for 24 hrs. Insoluble material was recovered by centrifugation (12,000x g, 20 min.), freeze dried, and labeled Ex2S or Ex2P.

Sugar Analysis.

Three milligrams of each fraction were suspended in water and sonicated to produce a uniform suspension. Sugar analyses were performed as follows: Hexose by the anthrone method (Dische, 1962) according to Fry (1988); Pentoses by H_2SO_4/orcinol method (Dische, 1962) according to Fry (1988); Uronic acids by the m-hydroxybiphenyl method (Blumenkrantz, 1973) according to Fry (1988); and 6-deoxyhexose by the cysteine/H_2SO_4 method (Dische, 1962) according to Fry (1988). All 6-deoxyhexose samples were incubated at 100°C for 10 min. to reduce interference from hexoses.

Protein Determination.

Protein was determined by converting μg nitrogen derived by Kjeldahl digestion (McDonald, 1978) into μg protein. 10 mg samples of wall material from each fraction were placed into 16x150 mm glass tubes followed by the addition of 1 ml of 0.1 N sulfuric acid and 0.5 ml concentrated sulfuric acid plus one selenized boiling chip. Ammonium sulfate (10 μg nitrogen per tube) was used as a standard. All tubes were heated on a micro-Kjeldahl heating apparatus to 120°C until the volume was approximately 0.5 ml. The temperature was increased to 320°C and held

there for 10-20 min. Tubes were removed from the heat, allowed to cool, and 100 μl of 30% H_2O_2 was added to oxidize excess carbon. Tubes were returned to 320°C until solutions became clear. After cooling, samples were diluted with water and neutralized with 10 N NaOH. Ammonium was assayed colorimetrically by a sodium nitroprusside, phenol and sodium hydroxide reaction (Balis, 1971).

The resulting μg of ammonia were converted to μg nitrogen and this value was converted to μg of protein using a conversion factor (μg protein/μg nitrogen) derived from averaging calculated values from published amino acid compositions of proteins extracted from plant cell walls. The calculated values for μg protein/μg nitrogen were as follows: 7.78, 7.30 (Ryden and Selvendran, 1990); 7.14 (Massiot, et. al., 1988); 7.63 (Qi, et. al., 1991); 7.46 (Kawasaki, 1989); 7.30, 7.10 (Li, 1990); 7.21 (Iraki et. al., 1989). The resulting average was 7.37±0.22 μg protein/μg nitrogen. The μg nitrogen from the colorimetric ammonium assays were multiplied by the 7.37 conversion factor to obtain μg protein.

Assay for β-galactosidase Activity.

β-galactosidase activity was measured by a procedure modified from Wallner and Nevins (1974). Ten milligrams of fresh weight tissue from pH 4.5 or 5.5 cultures was suspended in 985 μl of 100 mM sodium phosphate buffer at either pH 4.5 or 5.5. Fifteen microliters of 100 mM p-nitrophenyl-galactopyranoside dissolved in 100 mM sodium phosphate buffer (pH 5.5) was added to the reaction, vortexed briefly, and incubated at 34°C for 10 min. Following incubation, 1 ml of sodium carbonate was

added to stop the reaction and develop the nitrophenol color. Samples were then centrifuged at 2000 x g for 10 min. to pellet the tissue. The supernatant was removed and read at 400 nm on a Gilford Stasar II spectrometer. Controls were run in the same manner as above except 10 μl of water was substituted for the tissue.

Hydroxyproline Analysis.

Hydroxyproline was isolated according to Lamport (1984) as outlined by Fry (1988). Ten milligrams of each fraction were suspended in 2 ml of saturated $Ba(OH)_2$ (approx. 0.2 M) in teflon lined screw-cap tubes and hydrolysed under nitrogen at 110°C for 8 hrs. Suspensions were transferred to beakers and 25 μl of 1% bromothymol blue was added. Beakers were incubated with CO_2 (Na_2CO_3 plus HCl) until the indicator turned and stayed yellow. Samples were frozen then thawed and centrifuged for 10 min. at 2,500 x g. The supernatant was recovered and the pellet washed with 1 ml H_2O and centrifuged as above. The combined supernatants were acidified with 200 μl 0.1 M H_2SO_4 and passed through a 1.5 ml bed volume column of acid washed, water rinsed, Dowex-50 in a Pasteur pipette column. After loading the samples, the column was washed with 5 ml of H_2O, the washing rejected, and then eluted with 2 M ammonium hydroxide. Samples were evaporated in a desiccator, and the residues resuspended in 0.5 ml H_2O. Hydroxyproline was assayed by the method of Kivirikko and Liesmaa (1959) as outlined in Fry (1988). Dilutions of the $Ba(OH)_2$ hydrolysates containing 0.1-2.0 μg of hydroxyproline in 300 μl H_2O were mixed with 300 μl of ice-cold 0.36% (v/v) bromine in 1.25 M NaOH. Samples

were vortexed and incubated at 0°C for 10 min. With the samples at 0°C, the following were added in order with vortexing between each addition: 1.) 15 μl of 16% Na_2SO_3, 2.) 300 μl of 5% p-dimethylaminobenzaldehyde in 1-propanol, 3.) 150 μl of 6 M HCl. Samples were then incubated in a 95°C water bath for 2.5 min. followed by an incubation at 20°C for 20 min. and an absorbance reading at 560 nm.

Electron Microscopy.

Aggregated (pH 5.5) and non-aggregated (pH 4.5) tissue, grown semi-continuously for two months, was screened with a 310 μm mesh screen to obtain samples representative of >90% of the size classes in each culture. Approximately 1 mg FW of each tissue was placed in an Eppendorf tube and fixed in 6% glutaraldehyde in 0.05 M phosphate buffer at pH 6.8 for 8-12 hrs (Halperin and Jensen, 1967). The cells were then transferred to unbuffered 2% osmium tetroxide for 24 hrs. The tissue was dehydrated in a graded acetone series and stained in 1% uranyl nitrate in 70% acetone. Tissue was embedded in Spurr's resin, trimmed and sectioned with an ultramicrotome. Sections were placed on copper grids and examined on a Hitachi H-800 electron microscope.

Cell wall widths were measured on high contrast micrographs using a ruler with values recorded to the nearest 0.5 mm. Measurements were taken at regions of the wall which demonstrated no increase in width over at least 2 cm in length, and were not near any three cell junctions.

X-ray Microanalysis of Ca^{2+}.

X-ray microanalysis was performed on the Hitachi H-800 using a Link energy dispersive Pentafet X-ray detector with spectra acquired by the Link QX-2000 processor. For tissue samples, the following parameters were used: accelerating voltage 100 kV, 150 μm C_2 aperture, 200 sec. live time acquisition with the microscope in the STEM mode at 10 k magnification. Probe current was set to approximately 6.0 nA (measured from the grid bar). Three to seven independent cell wall spectra were obtained per cell. In addition, cytoplasmic and resin spectra were also acquired. The electron beam was in spot mode for all tissue spectra.

Calcium standards whose Ca^{2+} concentration had been verified by atomic absorption spectrometry were used to quantify cell wall Ca^{2+} content. Standards were composed of calcium naphthenate embedded in Spur's resin (Armstrong 1988), sectioned ultrathin, placed on copper grids, and carbon coated. The two standards, 110 and 195 mM Ca^{2+}/kg resin, were analyzed as above with the electron beam rastering over a small area. Four random, independent spectra of 2 sections of each standard were made. In addition, spectra from the copper grid were acquired with the probe current at 3 pA and C_2 aperture at 50 μm.

Section thickness measurements were obtained with a Gatan Model 607 Electron Energy Loss (EEL) Spectrometer. Three EEL spectra were obtained per section.

Calculation of Ca^{2+} Concentration.

To correct for varying background intensities, specimen spectra and standard spectra were scaled to each of their respective grid bar spectra using the Cu Kα peak. The grid bar spectrum was then subtracted from the specimen and standard spectra to obtain Ca Kα peak intensities minus contributions from Cu peaks. Peak to background values were then calculated using the corrected spectra for both specimen and standard. For background substraction, 20 eV windows were set on both sides of the Ca Kα peak (3.03-3.22 keV + 4.63-4.82 keV) and one 50 eV window where no obvious peaks were located (5.35-5.84 keV). The average background counts per channel was determined and the appropriate background counts were subtracted from the calcium window. Peak to background values were then corrected for differences in section thickness using values derived from EEL spectra taken with the specimen and standard spectra. The corrected values represent the relative mass fraction of Ca^{2+} in the specimen and the standard (C_i and $C_{(i)}$ respectively). By using the formula $C_A = (C_i/C_{(i)})C_{(A)}$, where C_A is the absolute mass fraction (concentration) of Ca^{2+} in the specimen analyzed and $C_{(A)}$ is the concentration of Ca^{2+} in the standard, the absolute concentration of Ca^{2+} in the cell wall can be calculated (Goldstein, 1981). Calcium concentrations for pH 4.5 and 5.5 cell walls were calculated and averaged with each standard to check the precision of the measurements and then all values for both standards were averaged to obtain the final Ca^{2+} concentration at each pH and then expressed as μmoles/mg DW to enable comparison to other published values. The expression of the Ca^{2+}

concentration per unit mass derives from the convention where the number of molecules measured is expressed per unit of material common to the samples and to the standards. If one assumes that a kg of resin occupies one liter and that the density of wall and resin are the same, the Ca^{2+} concentration units of mmoles/kg is equivalent to millimolar concentration. A problem arises if the density between samples and between samples and standard are not equal. In this case, if the density of wall material is different between 4.5 and 5.5 samples in a given volume, differences in Ca^{2+} concentration between 4.5 and 5.5 samples may be over or under estimated. If densities are assumed to be the same, the differences are accurate. In these experiments, we have assumed that the densities of the areas in each section being analyzed are not different enough to introduce significant error. We base this assumption on the fact that the chlorine peaks, derived from the resin in which the sample and standards are embedded in, are relatively uniform between samples and between samples and standards.

2-D Thin Layer Chromatography of Monosaccharides.

Ten milligram samples of each cell wall fraction were hydrolysed with 1.5 ml 2 M TFA for 1 hr. in an autoclave (121°C, 15 psi) and the supernatant, after a 10 min. centrifugation at 2000 x g, was evaporated to dryness in a desiccator under vacuum. The residue was suspended in 1 ml 95% ethanol and a volume was applied as a concentrated spot to the lower left-hand corner 1.5 cm from the bottom and 1.5 cm from the left edge on Whatman K2 Microcrystalline cellulose TLC plates to give

a concentration of 10-20 μg sugar. The solvent system used was a modification of Metraux (1982) and Fry (1989): first dimension, butanol/acetic acid/water (3:1:1) followed by ethyl acetate/pyridine/water (10:4:3); second dimension, phenol/water/formic acid (100:98:2, w/v/v organic phase only). Sugar spots were visualized by spraying the plates with aniline hydrogen-phthalate and heating with a hot air blower (Fry, 1988).

Gel Permeation Chromatography of Media CWM.

Gel permeation chromatography of CWM was performed according to Hohl et al. (1991). The column was 1.4x50 cm packed with Sephadex G-25 Medium. Eluent was 50 mM NaCl plus 0.01% (w/v) sodium azide. Fractions were collected at a flow rate of 14-15 ml/hr with a void volume of 34 ml. Fraction size was 10 drops/fraction or approximately 550 μl/fraction. Sugars were analyzed as described previously. Standards used included Blue dextran (2 million MW), inulin (5,000 MW), stachyose (667 MW), cellobiose (342 MW), and glucose (180 MW).

Methyl Esterification Analysis.

Methyl esterified galacturonic acid was reduced as described by Maness et al. (1990). Cell wall fractions (3 mg) were suspended in 600 μl 1 M imidazole-HCl buffer, pH 7.0 and placed on ice. 120 mg of sodium borohydride was added to the samples and cooled for 1 hr. Glacial acetic acid, 300 μl, was added to each tube to remove excess borohydride. An equal volume of water was added and the reduced

pectin precipitated by addition of 2 ml of 95% ethanol and recovered by centrifugation. The precipitate was resuspended in water (1 ml) and reprecipitated twice more with 95% ethanol and the residue lyophilized. Controls (non-reduced samples) were carried through the same procedure minus the addition of sodium borohydride. Uronic acids were assayed as described in "Materials and Methods".

3. RESULTS

Effect of pH on Growth of Aggregating Cell Types.

Clonal cultures were established to determine whether the pH's of the two media pH's were differentially effecting the growth of an aggregating or non-aggregating phenotype and thus causing the size class distribution seen in Figure 1. Ten clones were isolated as outlined in "Materials and Methods" and grown at pH 4.5 for 20 days in WCM-4 medium (Table 1). All clones except one showed a decrease in the larger size classes and a concomitantly large increase in percent dry weight in the smaller size classes consistent with observations of the heterogenous experimental cultures (Figure 1). Clone #28 shows a shift in aggregate size distribution (ASD) characterized by an increase in the >580 μm size class which is opposite to the other 9 clones. This clone may either have a different genotype and thereby produce an ASD at pH 4.5 opposite to what is expected, or exhibit the same phenotype but to a different degree, and that given more time, the ASD would have shifted to the expected pattern. None of the clones were grown at pH 5.5 so it is not known whether these clones would have reversed their distribution at the higher pH. Data from nine of the ten clones support the position that they parallel the behavior of the culture as a whole and respond to changes in pH in a manner consistent with the data in Figure 1.

Table 1. Effect of Growth at pH 4.5 on Size Class Distribution as a Percentage of the Sum of Dry Weights of All the Size Classes of Clones of Carrot Cell Line RWC-63.

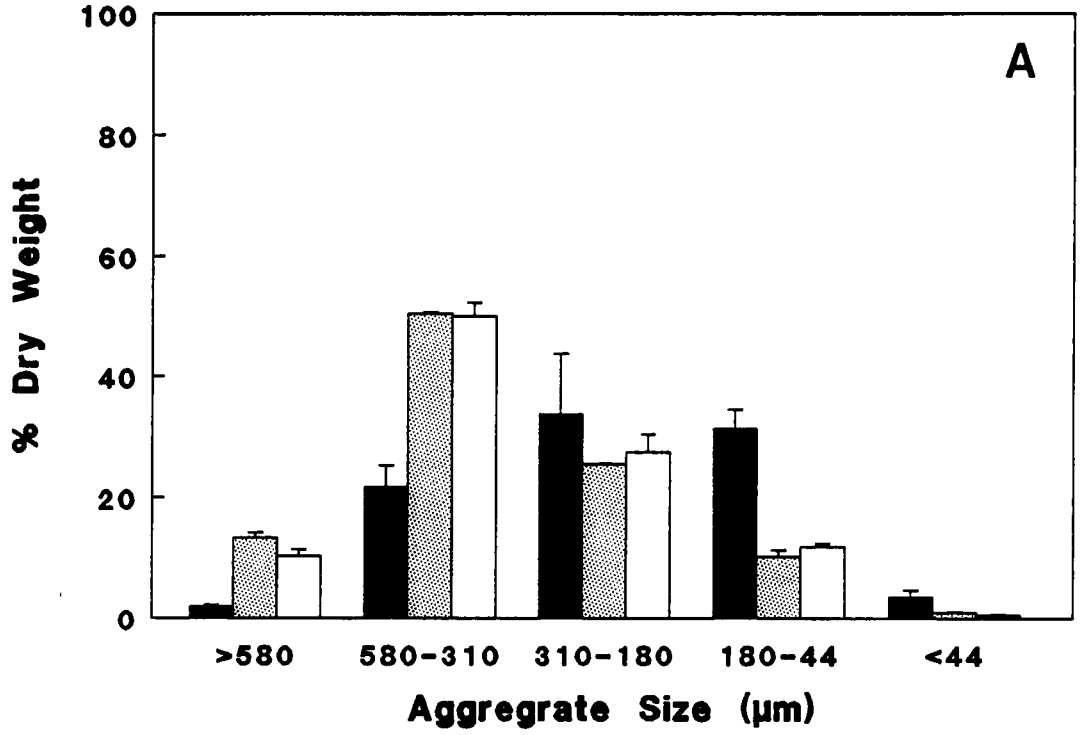
Clone #		Size Class[1]					Dry Wt.
		A	B	C	D	E	
							mg/ml
2	B	42.6[2]	22.0	14.1	18.0	2.9	4.657[3]
	E	24.2	22.2	20.6	32.9	0.6	1.016[4]
6	B	1.4	3.8	16.3	76.3	2.1	3.799
	E	0.0	1.0	8.4	87.9	2.5	0.779
16	B	3.3	6.0	22.8	65.9	1.8	4.529
	E	0.3	2.7	10.1	82.8	4.0	0.856
20	B	1.7	2.5	16.4	78.5	0.8	4.490
	E	0.0	1.2	12.4	85.3	1.0	0.889
21	B	5.3	12.5	28.7	51.4	1.9	4.461
	E	0.1	14.6	27.2	55.6	2.4	0.948
23	B	14.2	13.4	35.8	33.5	3.0	4.131
	E	0.0	17.3	36.3	44.2	2.2	0.799
27	B	0.8	2.8	20.4	74.4	1.4	4.066
	E	0.0	7.9	17.3	74.2	0.4	0.835
28	B	13.5	7.4	31.8	45.1	2.0	4.899
	E	18.8	17.4	20.5	40.0	3.2	0.876
29	B	3.3	4.7	16.3	73.9	1.7	4.453
	E	0.0	3.8	17.8	76.5	1.8	0.869
30	B	30.9	27.5	15.9	21.3	4.3	4.524
	E	0.0	33.3	21.7	39.4	5.5	0.789

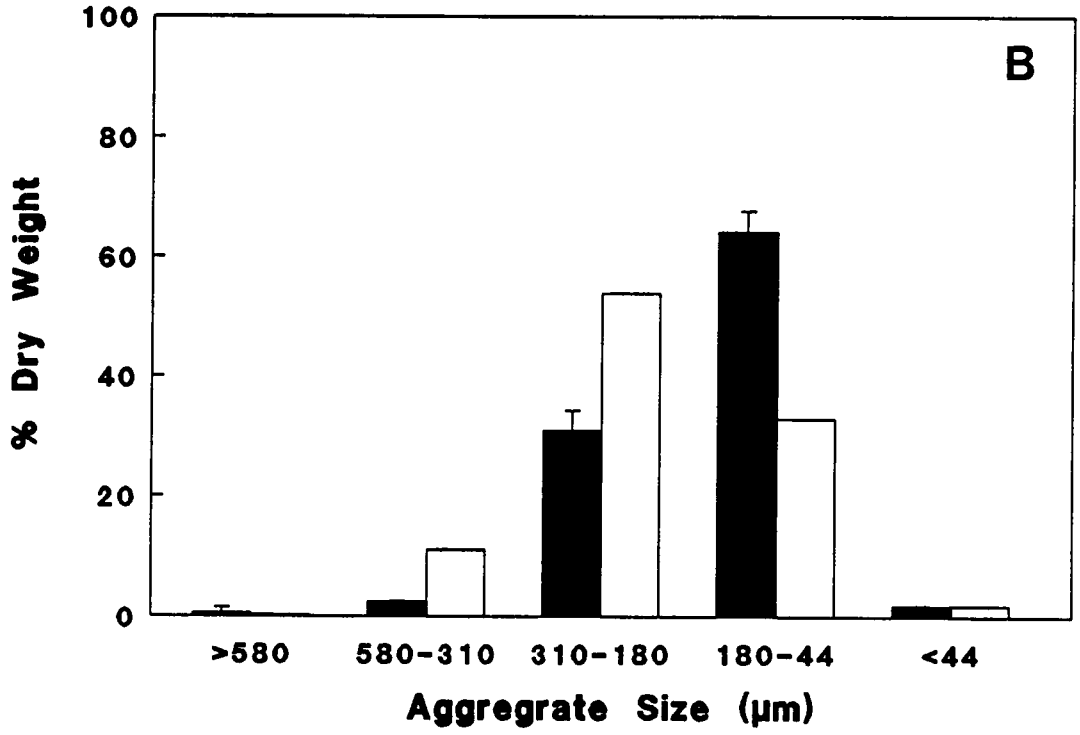
Cultures were grown semi-continuously at pH 4.5 for 20 days. Ending pH was within 0.005 units of starting pH of 4.5. [1]*A=>580μm, B=580-310μm, C=310-180μm, D=180-44μm, E=<44μm.* [2]*Values are means of single samples from replicate flasks.* [3]*Values derived from duplicate 10ml samples from batch cultures.* [4]*Values derived from duplicate 20ml samples from semi-continuous cultures. B=Beginning of culture period, E=End of culture period.*

Further, if the Selection Hypothesis was operating in the carrot cultures, one would expect clones with a high proportion of aggregates (such as clone #2, 28, or 30) to show a low or decreasing dry weight culture density. For instance, clone #28 shows an increase in % dry weight in the >580μm size class and a steady state culture density of 0.88 mg/ml showing that growth is just as in high as those clones which are disaggregated (clone #6, 16, 20, 27, or 29). This observation is not consistent with the Selection Hypothesis.

The question of whether the increase or decrease in size classes results from an increase or decrease of cell growth of a separate cell population or from the aggregation or disaggregation of cells in the entire culture is also addressed in Figure 3. Batch cultures at 2 and 3 pHs were inoculated with screened tissue from 2 month old semi-continuous cultures. Batch cultures were used in this instance due to low inoculum density. The screened tissue was of a particular size class (580-310 μm, Figure 3A and 180-44 μm, Figure 3B) which contained only cells and/or aggregates within that size range. In experiments where the batch culture pH was the same as the pH of the inoculum culture, the size class distribution of the aggregates both increased and decreased during culture (Figure 3A, pH 5.5; Figure 3B, pH 4.5). This increase in the range of size of aggregates may be due to culturing the cells by the batch methodology which would allow the accumulation of other sized aggregates since 20% of the culture is not removed daily as in semi-continuous culture. Alternatively, the shift in ASD may simply be a consequence of the dynamic behavior of the culture. In Figure 3A, aggregated tissue starting in the 580-310 μm size class

Figure 3. Size Class Distribution of Single Size Class Tissue Grown in Batch Culture. In A, cultures were inoculated with tissue composed of 580-310μm size aggregates derived from 3 month old, pH 5.5 semi-continuous cultures. In B, cultures were inoculated with tissue composed of 180-44μm size aggregates derived from 3 month old, pH 4.5 semi-continuous cultures. Both cultures were grown for a period of 21 days with subculture at every 7^{th} day. Black bars represent tissue grown at pH 4.5; stippled bars, pH 5.5; open bars, pH 6.5. The pH 5.5 cultures in B became contaminated. Values are mean$\pm$SE of three replicate flasks per pH per experiment.


A
100
80
60
40
20
0
% Dry Weight
>580
580-310
310-180
180-44
<44
Aggregrate Size (µm)


B
100
80
60
40
20
0
% Dry Weight
>580
580-310
310-180
180-44
<44
Aggregrate Size (µm)

showed an even greater shift in distribution toward the small size classes when cultured at pH 4.5, compared to the tissue cultured at pH 5.5 or 6.5 which remained largely at the 580-310 μm size class. Similarly, non-aggregated tissue, initially at 180-44 μm in size, showed some change in ASD when grown at pH 4.5, but showed much greater increase in the larger size classes when grown at pH 6.5.

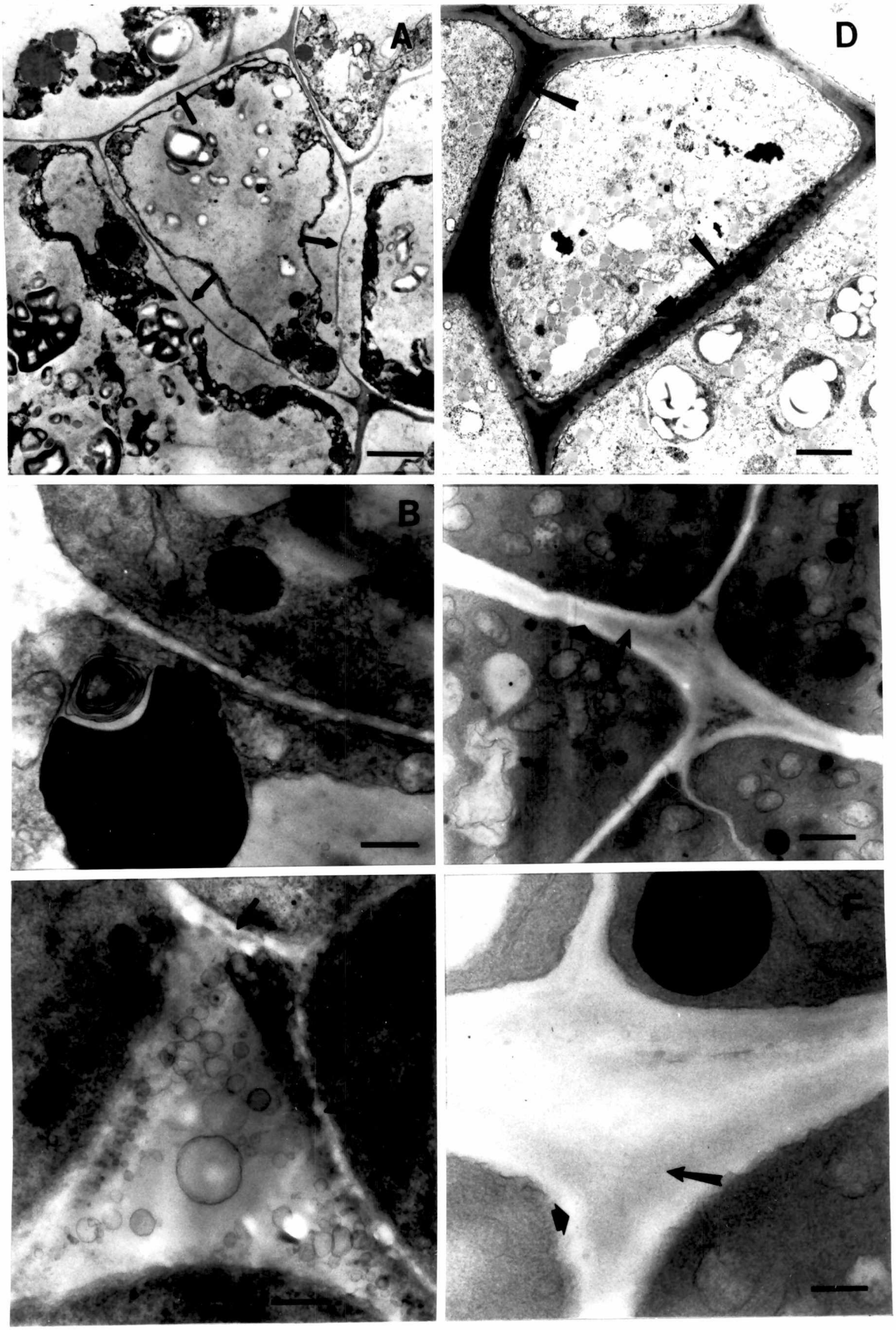
Taken together, the various lines of evidence discussed above suggest that the Selection Hypothesis is not valid. The changes in ASD seen at different culture pHs is the result of a response to pH rather than a growth based, genotypic selection.

Cell Wall Ultrastructure.

Figure 4 shows cells grown at pH 5.5 have a prominent middle lamella as well as extensive primary wall thickenings. Higher magnifications show the very distinct middle lamella at three cell junctions. In contrast, pH 4.5 grown cells exhibit thinner primary walls relative to pH 5.5 cells and no indication of a middle lamella at three cell junctions. Higher magnifications detail the absence of the middle lamella and the thinness of the primary wall. The micrographs reveal a striking difference between the two cell types.

Results of cell wall width measurements indicate pH 5.5 and pH 4.5 cells have a significantly different ($p < 0.001$) mean wall width of $3.078 \pm 1.635 \mu m$ (N=76) and $1.784 \pm 0.727 \mu m$ (N=102) respectively, indicating 1.7x more wall material is deposited between two neighboring cells grown at pH 5.5 than at pH 4.5.

Figure 4. Cell Wall Ultrastructure of Carrot Cells Grown at pH 4.5 and pH 5.5. Panels A-C and D-F depict cells grown at pH 4.5 and 5.5 respectively. Panels A and D were additionally stained with 1% uranyl nitrate. Panel A, low magnification of pH 4.5 cell showing general appearance of cell walls (small arrows) (4200x, bar = 2.4 μm). Panel D, low magnification of pH 5.5 cell showing general appearance of primary wall and middle lamella (short arrows and long arrows respectively) (6500x, bar = 1.5 μm). Panels B and E show details of walls. The small arrow (B) indicates the cell wall minus the middle lamella in pH 4.5 cells. pH 5.5 cells appear to have a distinct primary wall (short arrow) and middle lamella (short arrow) (B; 22,000x, bar = 0.45 μm. E; 12,000x, bar = 0.83 μm). Panels C and F detail the differences in four cell junctions between pH 4.5 cells (C) and pH 5.5 cells (F) with arrows as in B and E (C; 18,000x, bar = 0.55 μm. F; 25,000x, bar = 0.40 μm).


A
D
B

Cell Wall Calcium.

To determine the effects of medium H^+ ion concentration on the bound cell wall Ca^{2+}, carrot cells grown semi-continuously at each pH were fixed, sectioned, and examined by X-ray microanalysis. Table 2 shows that in cultures grown at pH 5.5, aggregates >360 μm in diameter and which comprised 90% of the culture, contained 3 fold more wall bound Ca^{2+} than pH 4.5 aggregates <360 μm in diameter and comprising 95% of the culture. Preliminary, less complete data than given in Table 2, indicated that pH 4.5 grown aggregates >360 μm in diameter have less wall Ca^{2+} than aggregates <360 μm in diameter and pH 5.5 grown aggregates <360 μm in diameter exhibited levels of wall Ca^{2+} comparable to pH 5.5 aggregates >360 μm in diameter (data not shown). This indicates that the effect of pH on wall bound Ca^{2+} is real and not due solely to differences in the size of the aggregates. Thus, cells grown at pH 5.5 and then transferred to pH 4.5 will have less wall bound calcium at the lower pH. The range of Ca^{2+} concentrations are ten fold higher than cell wall Ca^{2+} concentrations reported in pea stem (0.09--0.058 μmole Ca^{2+}/mg DW, Nakajima et al. 1981). The difference in levels is unresolved at this time.

To determine whether different calcium levels in tissue grown at the two pHs causes aggregation or disaggregation, carrot cells were grown semi-continuously and by batch methodology in the presence of various concentrations of chelators, and in separate cultures with higher than normal Ca^{2+} concentrations. If Ca^{2+} levels are critical to aggregation, the added chelators should remove Ca^{2+} and produce a change in aggregate size distribution similar to that of changing the medium pH from

Table 2. 2nd X-ray Microanalysis of Insoluble Cell Wall Ca^{2+}

Tissue	Wall Ca^{2+} Concentration
	μmoles Ca^{2+}/mg DW tissue
pH 4.5 <310μm aggregates	0.18±0.05
pH 5.5 >310μm aggregates	0.56±0.10

Values are mean±SD of 10 independent cell wall spectra acquired at 3 separate cells per section. Spectra were acquired and concentrations calculated as described in "Materials and Methods".

5.5 to 4.5. Supplying higher Ca^{2+} levels to disaggregated tissue at pH 4.5 would reverse this process by increasing the cell wall calcium, increasing pectin cross-linking and causing greater aggregation. The results (Table 3) show that regardless of the culture methodology or which chelator is used, removing the Ca^{2+} from the cell wall of aggregated cells grown at pH 5.5 does not produce a change in aggregate size distribution similar to that when the pH is switched from 5.5 to 4.5 (see bottom row in Table 3). In addition, a 10 fold increase in calcium concentration at pH 4.5, which in effect provides a H^+/Ca^{2+} ratio equal to that at pH 5.5, does not induce aggregation of disaggregated cells but does cause a ten fold decrease in >570 μm tissue and a two fold or more increase the 180-44 μm aggregates. This is opposite to the effect expected if Ca^{2+} is responsible for cell aggregation. The disaggregation effect of the 50 mM Ca^{2+} level may be due to non-physiological, ionic interactions within the wall which inhibit cross-linking between wall polymer molecules (Morris and Northcote 1970, Sentenac and Grignon 1981).

Clearly, cells grown at pH 4.5 have less wall bound Ca^{2+} than cells grown at pH 5.5, which correlates with disaggregation. However, if the Ca^{2+} is bound up by application of exogenous chelators, thereby lowering the wall Ca^{2+} concentration, the cells do not disaggregate to the same extent they would if transferred from pH 5.5 to pH 4.5. Likewise, adding Ca^{2+} at pH 4.5 does not increase the aggregate size to that found at pH 5.5. This evidence rules out a role for Ca^{2+} in the aggregation/disaggregation phenomena.

Table 3. Size Class Distribution of Carrot Cell Cultures Grown with EGTA, CDTA, and Elevated Levels of Ca^{2+}.

	Size Class (μm)					
Treatment	>570	570-310	310-180	180-44	<44	Total D.W.
			% D.W.			*mg/ml*
0mM EGTA	80±7	9±0	4±0	3±1	2±2	0.84±0.20
1.5mM EGTA	66±15	11±2	8±2	5±2	6±4	0.26±0.12
2mM EGTA	64±3	14±2	10±1	6±1	6±4	0.34±0.04
3mM EGTA	71±5	10±0	8±2	4±2	6±2	0.1±0.02
1mM CDTA	91±2	4±1	2±0	1±0	4±1	0.83±0.04
10mM CDTA	67±8	14±2	3±1	3±0	14±10	0.14±0.01
50mM CDTA	41±0	20±3	6±1	7±1	27±2	0.14±0.01
1.5mM Ca^{2+}*	10±3	17±3	33±2	40±1	1±0	0.90±0.08
15mM Ca^{2+}	1±0	4±0	9±0	85±7	1±0	0.74±0.06
50mM Ca^{2+}	0±0	1±0	3±1	94±5	2±0	0.66±0.04
5.5-->4.5	10±0	17±2	29±1	42±2	2±0	1.00±0.02

*EGTA and Ca^{2+} treatments were grown semi-continuously. CDTA treatment was grown by batch methodology subcultured every 7 days. All cultures were grown in WCM-4. pH of the treatments were as follows: EGTA--6.0, CDTA--6.5, Ca^{2+}--4.5. Cultures were grown for 30 days with the final pH within 2% of the initial pH. Values are mean±SD of 3 independent cultures per treatments. *Normal Ca^{2+} level in WCM-4 media. The switch culture (bottom row) was grown semi-continuously in normal WCM-4 media. Inoculum was from pH 5.5 SCC whose size class distribution was identical to that of the 0mM EGTA treatment.*

β-Galactosidase Activity.

The activity of β-galactosidase was measured in whole tissue to determine if pH affected the enzymatic activity such that it correlated with the disaggregation of cells seen at pH 4.5. Table 4 shows the activity of β-galactosidase of both pH 4.5 and 5.5 tissue at their normal culture pHs and at opposite pHs. pH 5.5 grown tissue showed higher levels of β-galactosidase activity than pH 4.5 grown tissue. More activity was measured at an assay pH of 4.5 than at pH 5.5 which is consistent with the pH 3.7 optima reported for β-galactosidase in sycamore cell cultures (Keegstra and Albersheim 1970). The activity of β-galactosidase has been shown to be localized at the surface of plant cells (Fry 1988). Regardless of assay buffer pH, pH 4.5 grown tissue showed lower activity suggesting that less enzyme is present in tissue grown at pH 4.5 relative to tissue grown at pH 5.5. This result does not correlate with the suggestion by Wallner and Nevins (1974) that the degradative activity of β-galactosidase is involved in the breakup of cell aggregates.

Cell Wall Carbohydrate Composition.

To identify differences in the composition of the cell walls, an extraction procedure (see Figure 2) was used to isolate various fractions of the wall and these fractions were then assayed for particular classes of sugars. The extraction sequence was adopted on the basis that calcium cross-links the pectin of the cell wall and controls the aggregation/disaggregation of the cells. Therefore, by using a chelator to extract the wall bound Ca^{2+} and solubilize the pectin, it would be possible fractionate

Table 4. β-galactosidase Activity of Carrot Suspension Cells.

pH of Culture	pH of Assay Buffer	β-galactosidase Activity Abs_{400nm}/mg FW/min.
5.5	5.5	0.0055±0.0003
5.5	4.5	0.0097±0.0005
4.5	5.5	0.0035±0.0002
4.5	4.5	0.0038±0.0003

Samples were derived from 60 day old semi-continuous cultures. Values are mean±SD of 3 independent samples.

the cell wall and to maximize differences in the composition of the fractions of the cell wall of cells grown at pH 4.5 and those grown at pH 5.5.

The fractions are divided into two major divisions; F, containing cell wall material and Ex, containing extracted material (starch and protein). In the fractions, the number "1" indicates CWM before treatment with DMSO and PAW, and "2", after treatment with DMSO and PAW. In the Ex fractions, "1" is material extracted by DMSO and "2", material extracted by PAW. "S" stands for soluble or solubilized CWM and "P" for pelleted CWM. The sugar composition of the F and the media CWM is presented in Table 5.

Little difference in carbohydrate composition is seen between pHs at which tissue was grown, in each of the fractions, while considerable difference exists between fractions from tissue grown at either pH's. In fractions F2S and F2P there is significantly ($p < 0.001$) higher hexose at pH 4.5 vs. pH 5.5, however, the relative size of the difference is not greater than 25%. Significant differences ($p < 0.001$) are present in the media CWM in pentoses and uronic acids both being higher at pH 4.5. The decrease in hexose content in the F2P versus F1P fractions reflects removal of starch. The starch content of the cells grown at pH 5.5 was 20% higher than that of cells grown at pH 4.5 calculated from the carbohydrate recovered in the DMSO extract of pH 4.5 and 5.5 (Table 6). Six times more protein in the F1S and F2S fractions than in the F1P or F2P fractions shows that the incubation with CDTA extracted most of the cellular protein into the F1S fraction. Though the micrograms sugar and protein per milligram dry weight increases in fraction F2S following DMSO

Table 5. Sugar Composition of Extracted Carrot Cell Wall Fractions.

Fraction		Hexose	Pentose	Uronic acid	6-deoxy hexose	Protein	Total μg Material Recovered
		μg/mg D.W. extracted cell wall fraction					
F1S	4.5	91±9	63±3	56±1	2±0	405±20	617±33
	5.5	81±5	67±2	65±2	4±0	455±13	672±22
F1P	4.5	687±26	204±8	147±7	31±3	64±4	1131±48
	5.5	736±12	173±6	126±2	25±2	76±1	1136±23
F2S	4.5	117±15	88±7	79±13	10±2	489±21	783±58
	5.5	88±12	97±2	74±9	10±1	584±13	853±37
F2P	4.5	495±16	294±14	189±5	45±4	62±1	1085±40
	5.5	447±25	300±7	188±5	44±3	69±5	1048±45
Media CWM	4.5	148±20	243±8	487±45	67±10	105±7	1150±90
	5.5	149±17	211±5	290±37	55±9	118±7	823±75
Total CWM	4.5	389±28	215±7	255±25	72±6	--	931±66
	5.5	398±18	216±3	245±35	78±8	--	937±64

Wall fractions were assayed as in "Materials and Methods". Protein was determined by Keljdahl digest and the μg nitrogen converted to μg protein as outlined in the "Materials and Methods". Values are mean±SD of 3 parallel experiments assayed in triplicate. Total CWM is oven dried tissue extracted with DMSO and PAW and suspended in H_2O and assayed. Dashes = not assayed.

Table 6. Dry Weight Material Recovered from DMSO and PAW Extraction of F1 Carrot Cell Wall Fractions.

Fraction		Initial wt.	Resulting F2 Fraction	Treatment	
				DMSO	PAW
			mg D.W.		
F1P	4.5	50.0	33.5±2.5	18.3±1.1	Trace
	5.5	50.0	30.6±6.3	22.0±2.0	Trace
F1S	4.5	50.0	21.5±0.5	Trace	Trace
	5.5	50.0	23.0±0.5	Trace	Trace

Values are mean±SD of 3 independent samples assayed in triplicate. Trace = < 1 mg carbohydrate recovered.

and PAW treatment, only 80-85% of the total fraction weight of F2S is accounted for. Higher than expected total composition values in the F1P and F2P fractions are due to the increase in mass (10% for hexoses, 12% for pentoses) in the form of water upon hydrolysis of the sugars.

From the data in Table 5, the percent composition of the walls was calculated based on the total weight of material accounted for by the analyses. The result is Table 7 which again shows the percent sugar and protein content between pHs of each fraction is very similar. The media CWM shows higher uronic acid at pH 4.5 but less hexose. Also, the percentage amounts for the sugar classes are different in the media CWM than in the fractions of the wall proper with the majority of the sugar found in the media CWM being pentose and uronic acid rather than hexose.

To obtain the F2 fractions, 50 mg of F1 fractions were treated with DMSO and PAW and the mass recovered from both treatments (Table 6). In the F1P fraction most of the extractable material was in the form of starch removed by DMSO treatment. Upon sugar analysis of the DMSO extracted material, greater than 96% was hexose with the remaining divided between the other three classes of sugars. The F1S fraction behaved quite differently in that 55-60% of the mass could not be recovered in either the DMSO or PAW treatments (see Table 6). Since starch in the F1P fraction could be recovered from the DMSO treatment by ethanol precipitation, the missing F1S material was thought to be in the PAW extract and not recoverable by precipitation with 70% (v/v) ethanol.

The material in the F1S and F2S fractions that could not be identified as sugar

Table 7. Composition of Extracted Carrot Cell Wall Fractions Expressed as a Percent of Total μg of Material Recovered.

Fraction		Hexose	Pentose	Uronic Acid	6-deoxy hexose	Protein
		% sugar or protein in total sample recovered				
F1S	4.5	14.7±1	10.2±0	9.1±0	0.3±0	65.6±3
	5.5	12.0±1	10.0±0	9.7±0	0.6±0	67.7±2
F1P	4.5	60.7±2	18.0±1	13.0±1	2.7±0	5.6±0
	5.5	64.8±1	15.2±1	11.1±0	2.2±0	6.7±0
F2S	4.5	14.9±2	11.2±1	10.1±2	1.3±0	62.4±3
	5.5	10.3±1	11.4±0	8.7±1	1.2±0	68.5±2
F2P	4.5	45.6±1	27.1±1	17.4±0	4.1±0	5.7±0
	5.5	42.6±2	28.6±1	17.9±0	4.2±0	6.6±0
Media CWM	4.5	17.6±2	29.0±0	43.0±4	0.9±0	9±1
	5.5	20.4±2	28.4±1	37.9±5	1.4±0	12±1
Total CWM	4.5	41.7±3	23.1±1	27.4±3	7.7±1	--
	5.5	42.5±2	23.1±1	26.1±4	8.3±1	--

Percentages are calculated by dividing μg sugar or protein in each fraction by the total μg assayed in each fraction (see Table 5).

was thought to be protein (both soluble and complexed) which had been extracted in the CDTA treatments when the tissue was initially fractionated. Estimation of protein content with Bradford's (Bradford 1976) reagent indicated very little protein in any of the fractions. This result could be explained if a large proportion of the protein was complexed with the CWM which would render it undetectable by the dye binding assay. To derive an accurate estimation of the protein in the F1S and F2S fractions, samples were digested with acid and the resulting nitrogen converted to μg protein (Table 8 and Table 9). Since the F1S and F2S fractions had previously been dialyzed thus removing any small molecular weight substances which might contain nitrogen (such as CDTA), it was likely that the nitrogen recovered was derived primarily from proteinaceous material, though other high molecular weight molecules (DNA, RNA, etc.) would also be present. Apparently, one treatment with PAW was insufficient to remove all protein from these wall fractions or the protein is covalently bound in the cell wall.

Table 10 shows the amount of material obtained from the semi-continuous cultures. Values were derived by averaging the fresh weight, the extracted dry weights recovered in F1S, F1P and the Media CWM and dividing by the milliliters of culture from which they were derived (300 ml). The values are based on a ml of culture to enable direct comparison between pHs. The pH 5.5 cell wall fractions F1S and F1P show 20 and 30% greater mass respectively. When combined together, 26% more material was recovered from pH 5.5 cells than pH 4.5 cells. In contrast, 23% more cell wall material was obtained from pH 4.5 medium than pH 5.5 medium and 30%

Table 8. Total Nitrogen by Keljdahl Digestion of Carrot Cell Wall Fractions.

pH	Fractions					
	Total tissue	F1S	F2S	F1P	F2P	Media
	μg nitrogen/mg dry wt. fraction					
4.5	28.4±2.9	54.9±2.7	66.3±2.8	8.7±0.5	8.4±0.2	14.3±0.9
5.5	26.9±1.0	61.8±1.7	79.2±1.7	10.3±0.2	9.4±0.7	16.0±1.0

Digestions were performed as in "Materials and Methods." Data are mean±SD of three samples assayed in triplicate.

Table 9. Protein Content of Carrot Cell Wall Fractions.

pH	Total Tissue	Fractions				Media
		F1S	F2S	F1P	F2P	
		μg protein/mg dry wt.				
4.5	209	405	489	64	62	105
5.5	198	455	584	76	69	118

Protein content calculated by multiplying the derived conversion factor 7.37 (see text) by the μg nitrogen/mg values in Table 8.

Table 10. Recovery of Material from Carrot Cell Cultures. Expressed as Weight of Tissue, Cell Wall Fractions, and Media Cell Wall Material per Unit Volume of Culture.

Culture	Total FW[1]	F1S	F1P	F1S+F1P	Media CWM
			mg d.w./ml culture		
pH 4.5	7.4±0.16	0.28±0.01	0.32±0.01	0.60±0.02	0.061±0.003
pH 5.5	5.2±0.09	0.35±0.01	0.46±0.003	0.81±0.01	0.047±0.010

Values are mean±SD (N=6) of 2 independent experiments each consisting of 3 separate 100ml cultures. [1]*mg FW/ml culture.*

more fresh weight tissue was present at pH 4.5 than at pH 5.5. The fact that 26% more cultured material (F1P and F1S) is found in pH 5.5 cells indicates that cells grown at pH 5.5 are synthesizing more cellular material than cells grown at pH 4.5. The amount of material recovered in the F2 fractions between pH 4.5 and 5.5, after treatment with DMSO and PAW, is almost the same (Table 6). The differences in the sum of mg dry weight of all fractions recovered from the tissue are real and these differences reflect the higher amount of cell wall material deposited by the cells grown at pH 5.5 and are consistent with the photomicrographic data of Plate 1 showing greater wall thickness at pH 5.5.

In addition to carbohydrate composition, the identity of sugars present in each of the fractions was investigated through TLC (Table 11). Glucose, galactose, arabinose, xylose, and galacturonic acid were present in every wall and media fraction. Rhamnose was only detected in the pH 5.5 soluble fractions. In addition, glucose (most likely from starch), arabinose and xylose were found in some of the Ex fractions as contaminants. Mannose and glucuronic acid were not detected in any of the fractions. The results are consistent with other published data and indicate no particular sugar is missing from the cell wall or media.

Degree of Methyl Esterification.

To examine differences in the degree of methyl esterification (DOM) of the wall pectins between cells grown at pH 4.5 and 5.5, samples were reduced with sodium borohydride and the change in uronic acid content measured (Maness et al.

Table 11. TLC Sugar Analysis of TFA Hydrolysed Carrot Cell Wall Fractions.

Fraction	Glu	Gal	Mann	Arab	Xyl	Rham	GalA	GluA
F1S 4.5	+	+	-	+	+	-	+	-
5.5	+	+	-	+	+	+	+	-
F1P 4.5	+	+	-	+	+	-	+	-
5.5	+	+	-	+	+	-	+	-
F2S 4.5	+	+	-	+	+	-	+	-
5.5	+	+	-	+	+	+	+	-
F2P 4.5	+	+	-	+	+	-	+	-
5.5	+	+	-	+	+	-	+	-
Ex1S 4.5	+	-	-	+	-	-	-	-
5.5	+	-	-	+	-	-	-	-
Ex1P 4.5	+	-	-	+	+	-	-	-
5.5	+	-	-	-	-	-	-	-
Ex2S 4.5	+	-	-	+	-	-	-	-
5.5	+	-	-	+	-	-	-	-
Ex2P 4.5	-	-	-	-	-	-	-	-
5.5	-	-	-	-	-	-	-	-
4.5 Media CWM	+	+	-	+	+	-	+	-
5.5 Media CWM	+	+	-	+	+	-	+	-

+ Indicates presence of sugar by visualization after detection with aniline reagent.
- Indicates no detection.

1990). The results, Table 12, indicate that the average degree of methylation in the cell wall fractions was consistently less than or equal to 20% which is in good agreement with DOM values for cotton, barley, and wheat cell wall preparations (Maness et al. 1990). However, absolute values and differences between pH 4.5 and 5.5 cell walls could not be reliably estimated due to the high degree of variability between sets of experiments. The cause of the variability is unknown. The method has demonstrated DOM values for cotton, barley and wheat wall samples with coefficients of variation (CV) greater than 20% of the mean (Maness et al. 1990). Apple pectin, with less than 10% methyl esterification, showed CVs greater than 40% which would seem consistent with the high amount of variability seen with the carrot samples. However, various purified, commercial apple and citrus pectins, with DOMs comparable to the cell wall preparations, showed CVs of only 2-4% of the mean (vs. 20% with whole cell walls). In addition, apple pectin samples analyzed in our lab showed a methyl content of 53±6% (CV of 11%, N=6), within 5% of the calculated DOM of 50% indicating that the method was being performed correctly. The above data suggests that the variability seen with this assay may be connected with the complexity or heterogeneity of the sample rather than with the DOM of the sample or some procedural error. Recently, Kim and Carpita (1992) devised an extension of the Manness protocol using sodium borodeuteride plus activation of free uronic acid with carbodiimide. They also showed the greatest standard error at DOM 30% or less. Neither Maness et al. (1990) or Kim and Carpita (1992) give an explanation for the high variability seen in their cell wall preparations. Alternative methods to

Table 12. Degree of Methyl Esterification of Carrot Cell Wall Fractions.

Fraction	pH	Experiment #				Average
		I	II	III	IV	DOM
		DOM %[1]				
F1S	4.5	-7.2	19.1	25.7	-1.4	9.1±15.8
	5.5	17.3	11.3	26.9	2.5	14.5±10.3
F1P	4.5	18.7	17.3	36.3	8.8	20.3±11.5
	5.5	16.9	13.1	7.6	5.9	10.9±5.1
F2S	4.5	-	20.0	22.3	10.7	17.6±6.1
	5.5	-	19.9	23.2	1.3	14.8±11.8
F2P	4.5	-	15.7	25.5	13.5	18.2±6.4
	5.5	-	1.7	18.3	3.1	7.7±9.2
Media CWM	4.5	13.1	10.4	32.1	7.5	15.8±11.1
	5.5	31.7	27.8	-2.5	15.6	18.1±15.4

[1]*Percentage of reduced galacturonic acid to native galacturonic acid. Experiments II, III, and IV were done in parallel and assayed at the same time. Values for experiments I, II, III, and IV are mean of triplicate samples whose SD≤5% of the mean. Dashes = not measured.*

determine percent DOM, such as titration with polycations (Mizote et al. 1975) or measurement of uronic acid and methanol contents (McFeeters and Armstrong 1984), were not tried and this question awaits further investigation.

Molecular Size of CWM in Carrot Cell Culture Medium.

Extracellular carbohydrate material was separated by gel permeation chromatography to characterize the size of the wall polymers released into the medium. In media from semi-continuous cultures at pH 4.5, hexose is found in both the monomeric and high MW fractions, whereas the classes of sugars which would be characteristic of wall polymers (pentoses, uronic acids, and 6-deoxyhexoses) and not of media components (sucrose or its breakdown products, glucose and fructose) are found exclusively in the high molecular weight fractions (data not shown). Though media CWM from the pH 5.5 semi-continuous cultures was not analyzed as extensively, pentoses were found exclusively in the high molecular weight fractions. This indicates that if incubation of the cells at pH 4.5 is causing release of wall components into the medium, it all released as high molecular weight material.

Cell Wall Hydroxyproline Content.

Hydroxyproline was extracted from various fractions and assayed to determine differences in bound hydroxyproline between cells grown at the two pHs. In all fractions analyzed, hydroxyproline content was from 162% to 26% higher in pH 5.5 cells relative to pH 4.5 grown cells (Table 13). Substantial amounts (approx. 5 times

Table 13. Hydroxyproline Content of Carrot Cell Wall Fractions.

	Total Tissue	Fractions			Media
pH		F1S	F2S	F1P	
		μg/mg dry wt. fraction			
4.5	0.41±0.01	0.61±0.05	0.74±0.05	0.16±0.02	5.24±0.59
5.5	0.82±0.11	0.99±0.06	0.93±0.05	0.42±0.09	5.48±0.34

Samples were extracted and assayed for hydroxyproline as in "Materials and Methods." Data are mean±SD of three or four independent samples assayed in triplicate.

that of the highest fraction) of hydroxyproline were found in the media CWM although the difference between pH 4.5 and 5.5 media was only 4%. The tissue and cell wall fractions from tissue grown at pH 5.5 all contain more hydroxyproline on a dry weight basis than do the tissue and fractions grown at pH 4.5.

4. DISCUSSION

The results of the aggregate size distribution of carrot clones grown at pH 4.5 demonstrate that cultures derived from single cells isolated from the heterogenous culture population display, for the most part, the same pH/aggregation pattern as do the experimental cultures. With only ten clones selected, it may be possible that genotypes which could display the growth characteristics of the Selection Hypothesis could still be present. The fact that one of the ten clones demonstrated aggregation at pH 4.5 seems to support this possibility. The most compelling evidence against the Selection Hypothesis is from the data in Figure 3 which shows that every size class can be derived from tissue comprising a single size class without a change in H^+ ion concentration. This proves that the changes in different aggregate sizes upon growth of carrot cells at different pH's arises from the breakup or maintenance of the cell aggregates. This implies a direct effect of pH on either the cell wall material itself or on the synthesis of this material, and not on the rate of growth of individual aggregating genotypes.

Electron micrographs of thin sections of both aggregated and non-aggregated cells show that cross-sections of the cell walls of aggregated tissue grown at pH 5.5 have greater width and a more pronounced middle lamella than walls of cells grown at pH 4.5. Quantification of wall width from electron micrographs shows pH 5.5 cells have a 1.7x greater mean wall width than pH 4.5 cells with the implication that 1.7x

more wall material is synthesized and bound into the wall in cells grown at pH 5.5. This apparent increase in wall material is reflected in the amount of wall material recovered from semi-continuous cultures at pH 4.5 and 5.5 (Table 10). The difference in wall material between pH 4.5 cultures and pH 5.5 cultures (derived from the addition of the F1P and F1S fractions in Table 10) is less (1.35x) than what would be expected based on thickness calculations from the electron micrographs (1.7x) and may be due to the different methods by which the numbers were derived, one measuring the width of a small number of cells the other measuring the quantity of wall material from a larger number of cells. The electron micrographs do appear to show less middle lamella, and thus less pectin in pH 4.5 cell walls, suggesting H^+ ions are preferentially effecting a single component of the cell wall. This selectivity could be explained by the fact that more total wall is deposited at pH 5.5, thus increasing the ability to visualize the middle lamella region. This interpretation is supported by the composition data (Table 7) which shows very little difference in wall uronic acids (pectin monomers) between pH 4.5 and 5.5 cells. Though the difference in wall width is not derived from all cells in an aggregate, and there may be differences in the wall width depending on where the cell is located in the aggregate, the 180 observations demonstrate that the walls grown at the two pHs are significantly different in gross morphology.

It is clear from Table 2 that cells grown at pH 4.5 contain significantly less (1/3) wall bound Ca^{2+} than cells grown at pH 5.5 which correlates with data showing lower amounts of wall material recovered from pH 4.5 cells. However, it was also

thought that the lower Ca^{2+} at pH 4.5 may have resulted from H^+ ion exchange with the Ca^{2+} at the lower pH. No data points to one mechanism or the other for lower Ca^{2+} levels in the cell wall. However, Table 3 clearly shows that chelation of calcium ions from the cell or an increase in the Ca^{2+} concentration such that the H^+/Ca^{2+} ratio is the same at pH 4.5 and at pH 5.5 is not sufficient to produce the cell separation seen in cultures transferred to pH 4.5 and thus shows that Ca^{2+} is not responsible for aggregation or separation of carrot cells in culture.

The fact that Ca^{2+} is not responsible for the aggregation states seen in the carrot cultures is not unique. There is evidence that Ca^{2+} release or exchange is not sufficient to cause wall loosening in other systems (Jarvis et. al. 1984, Virk and Cleland 1988) and therefore would not be able to create the aggregate states seen in the carrot cultures. Jarvis (1982) has shown that after removal of Ca^{2+} with CDTA from cell wall preparations of celery, cress, cucumber, and tomato, 60-70% of the total polygalacturonic residues remained with the cell wall. CDTA treatment of our carrot cells shows 59% and 61% of the uronic acids remained in the F2 fraction for pH 4.5 and 5.5 tissue respectively. Further, loosening of boiled (to inactivate wall enzymes) soybean cell walls by prolonged acidic treatments after removal of Ca^{2+} suggests that linkages other than Ca^{2+} bridges do exist and play a role in cell wall loosening (Virk and Cleland 1988, Rayle 1989). Removal of wall Ca^{2+}, by whatever process, is not a mechanism for wall loosening or stiffening and appears to play a minor role, if any, in the separation or formation of aggregates in carrot cell cultures. If coupled with some other wall loosening process, changes in

wall Ca^{2+} concentration could possibly effect the wall structure enough to cause aggregate formation or separation. For instance, Demarty et al. (1984) hypothesize that Ca^{2+} displaces protons on uronic groups, modifying the wall pH and thus changes the activity of wall enzymes which may inhibit or enhance wall expansion.

The effect of elevated levels of Ca^{2+} on aggregation is more difficult to explain. Increased levels of wall calcium have been shown to stiffen the wall matrix (Knee 1982, Stow 1989). The inhibition of cell elongation by exogenous Ca^{2+} appears to result from the inhibition of enzymes involved in wall loosening (Rayle and Cleland 1977). Results with carrot cells show greater disaggregation at higher Ca^{2+} concentrations though the highest concentration tested was significantly less than that of Knee (1982) or Stow (1989). One possible explanation is an ionic effect generated by the high levels of Ca^{2+}, disrupting the electrostatic field of the uronic acids which are responsible for the high affinity of Ca^{2+} for pectins (Sentenac and Grignon 1981). More simply, the high concentration of Ca^{2+} may be saturating all the possible calcium binding sites on the polygalacturonic acid residues rendering the polymer unable to bind or cross-link with other pectic polymers. The significance of the greater disaggregation seen at high Ca^{2+} concentrations in terms of a mechanism for aggregation in carrot cell cultures is unclear at this time.

Concerning, the activity of β-galactosidase, on an equal FW basis, 30% more activity is detected in cells grown at pH 5.5 than cells grown at pH 4.5. This activity is pH dependent with the activity in pH 5.5 grown cells doubling when the cells are assayed at pH 4.5 compared to the cells being assayed in buffer at pH 5.5. The

absorbance values indicate more enzyme is present in cells grown at pH 5.5 than at pH 4.5. The lower activity associated with the disaggregated cells at pH 4.5 is in contrast to the parallel increase in β-galactosidase activity and cell separation shown by Wallner and Nevins (1974). The increase in activity Wallner and Nevins saw may be related to the cells entering the stationary phase of the growth curve and thereby becoming stressed. The pH optima of the enzymes was not measured, so it is not known whether their activity was influenced by changes in the culture pH. The lack of correlation of β-galactosidase activity verses aggregation in the carrot cultures is not unexpected as it has been shown that inhibition of β-galactosidase by its galactolactone, does not block acid-induced growth (Evans 1974) suggesting that the activity of β-galactosidase may not be involved in acid-induced, wall loosening.

It must be stressed that involvement of other wall enzymes may be possible. Endo-(1-->4)-β-glucanase, which degrades xyloglucan a hemicellulose common in dicotyledonous plants, promotes the release of water soluble xyloglucan in pea stems upon incubation in acidic (pH 4) buffer (Jacobs and Ray 1975). Acidic pH has also been shown to induce a decrease in the mass-average molecular weight of xyloglucan (Nishitani and Masuda 1982). The pH optima for this enzyme is 5.5-6.0 and its activity is increased only 1.3 fold at optimum pH compared with neutral pH (Byrne et al. 1975). This suggests that the enzyme activity may not be significantly different at the two pHs at which the carrot cells are grown, indicating that endo-(1-->4)-β-glucanase may not be involved in the process of aggregation\disaggregation.

It cannot be determined from the sugar composition analysis whether

xyloglucan is released into the medium. The proportion of hexose to pentose in xyloglucan is 5:3 (Hayashi 1989) whereas the hexose:pentose ratio in carrot media CWM is 2:3. However, if other blocks of cell wall components were released into the media as a consequence of xyloglucan degradation, the characteristic xyloglucan ratio could not be obtained using only the colorimetric assays for sugars. This same scenario would hold true for β-(1,3)-glucanase (cellulase).

From the sugar composition data of Tables 5 and 7, there is very little difference in carbohydrate or protein composition between the walls of carrot cells grown at pH 4.5 and 5.5. Differences in individual sugars within each class may be present but undetected. The one exception is a higher percentage of hexose in pH 4.5 F2S and F2P fractions which may reflect a lower amount of other wall components in the wall. This is complemented by the significantly higher amount of uronic acid in pH 4.5 media, particularly in relation to the low amount of hexose found in the media. The percent composition of sugars found in the media is in general agreement with that of extracellular CWM from media of sycamore cultures (Burke et al. 1974), with the exception that 80% more uronic acid is found in the media of carrot cells. The decrease in the amount of hexose and the increase in the amount of pentose and uronic acid in carrot media CWM is in general agreement with that reported for media CWM of sycamore suspension culture cells (Burke et al. 1974).

Results from the gel permeation chromatography indicate that the CWM from the media is composed of polymer material with a small amount of hexose monomer

(probably originating from the WCM-4 medium). Together with the high percentage of pentose and uronic acid, the data indicates that the polymers being released into the media are high molecular weight polymers primarily composed of galacturonic acid and pentose sugars. Possible candidates for these high molecular weight polymers include homogalacturonan, consisting almost entirely of galacturonic acid, rhamnogalacturonans I and II, consisting mainly of galacturonic acid with a small proportion of rhamnose, and the hemicellulose, xyloglucan. The high molecular weight material in the medium suggests that whatever mechanism is operating in cell separation at pH 4.5 it is not a degradation of polymers to monomers with subsequent release into the medium.

In addition to polymer release, a relatively large amount of hydroxyproline is found in the media, though the percent difference between the two pHs is very small. Apparently, the high media hydroxyproline content is normal in carrot tissue (Chrispeels et al. 1974). More significant, is the two fold less hydroxyproline in total tissue from cells grown at pH 4.5 compared to tissue grown at pH 5.5 together with the observation that all fractions from tissue grown at pH 4.5 have lower hydroxyproline than tissue grown at pH 5.5. Though there is no direct evidence that the hydroxyproline isolated and assayed here is that of extensin, hydroxyproline is very characteristic of this molecule (Cassab and Varner 1988) and extensin has been isolated from carrot tissue (Stuart and Varner 1980, Showalter and Varner 1987). Hydroxyproline containing glycoproteins (HPCGs), such as extensin, play a major role in strengthening the wall via cross-linking to itself and to other polymers (Lamport

and Epstein 1983, Fry 1988, Varner and Lin 1989). One role is in forming an independent network of extensin molecules cross-linked to each other by isodityrosine bridges (Fry 1986). The cross-linking of *in vitro* extensin has been shown to be pH dependent (Cooper and Varner 1984). The mechanism for pH control could involve changing the enzymatic activity that cross-links extensin molecules via isodityrosine bridges. Alternatively, protonation of the histidine residues (11% of the molecule) would increase the cationic affinity of extensin for anionic residues of pectin thus prohibiting the cross-linking oxidation of tyrosine to other extensins (Cooper et al. 1984). Presumably, as a result of the lack of cross-linking at the lower pH, the network would loosen and extensin could be released into the media possibly attached to pectin or other polymers no longer held in place by the extensin network.

HPCGs, along with other CWM, have been found in the culture medium of tobacco cell cultures and identified by amino acid composition as extensin (Kawasaki 1989). Extracellular HPCGs of sycamore culture media have also been characterized and shown to be structurally different from wall bound HPCGs (Pope 1977, Keegstra et al. 1973) indicating that extensin may undergo modification upon release. The activity of peroxidase has been suggested as a mechanism which could control the formation of cross-links between extensin molecules (Epstein and Lamport 1984, Fry 1982, Cooper and Varner 1984). The pH optima for this peroxidase is between 5 and 7, allowing oxidative cross-linking to occur at higher pH, while at acidic pH, the activity and thus the ability to cross-link is inhibited (Pedreno et al. 1989). This enzyme control scheme could account for the aggregation states seen in the carrot

cultures and is supported by the lower hydroxyproline content of pH 4.5 cell walls.

The results from the data presented, indicate that the primary effect of medium pH with respect to cell aggregation/disaggregation involves the cell walls of carrot cells. Changes in wall Ca^{2+} concentration do not cause the pH induced change in cell aggregation. Growth of carrot cells at pH 4.5 has been shown to decrease the amount of wall material present in the wall without a significant change in the carbohydrate composition of either cell wall. The amount of specific wall components or fractions have been shown to decrease and these include the major classes of sugars characteristic of wall polymers, activity and possibly amount of β-galactosidase, wall bound Ca^{2+}, and hydroxyproline content. In addition, a higher amount of high molecular weight carbohydrate polymer (but not of protein or hydroxyproline) is found in the media of pH 4.5 cultures than in pH 5.5 cultures.

Two mechanisms are apparent which could account for the above results. The mechanisms involve a pH dependent inhibition or decrease in the rate of synthesis of wall material, or a pH dependent inhibition or decrease in the rate of incorporation of wall material into the wall. Both mechanisms take into account that the data argue against a rapid chemical or physical mechanism that requires much less time than the minimum two weeks and several generations of growth to achieve a significant change in the ASD of the carrot cultures. The first mechanism could involve a decrease in the rate of synthesis of cellulose or callose at the plasmalemma or reduction in the rate of synthesis of matrix wall components at pH 4.5. The decrease in rate of synthesis of matrix components would mean a decrease in wall

components such as matrix polysaccharides (polygalacturonic acids, rhamnogalacturonans, xyloglucans, etc.), Ca^{2+} bound to the matrix polymers, and hydroxyproline containing glycoproteins and other cell wall proteins as well as other wall components. The second mechanism involves a decrease in the rate of incorporation and would entail a decrease in the binding of the various wall components upon synthesis or release from endo-vesicles at the low pH. Lowering medium pH below 6.0 has been shown to decrease the rate of endo- and exo-cytosis in cultured mammalian cells (Cosson et al. 1989). The incorporation of cell wall material would be non-specific and account for the lack of difference in wall composition between pH 4.5 and 5.5 cells. One other possibility is some combination of the two which would comprise a decrease in the rate of synthesis of matrix polysaccharides which would produce less wall material at pH 4.5 plus a decrease in the incorporation rate of wall components which would result in release of polymers into the media at pH 4.5. The specific sequence of events resulting from the effect of pH on the cell wall cannot be deduced from the results presented here, but it is clear that the mechanism involves a pH-induced general reduction of constituents which comprise the plant cell wall. In addition, the data suggest that at pH 4.5 the synthesis and\or transport of the components to the cell wall which comprise the extensin network, such as HPCGs, is reduced such that a new or additional extensin network cannot be assembled in the cell wall. This would cause an overall loss in wall strength and result in a decrease in total wall polysaccharide as a portion of the wall was released into the medium. Such a hypothesis is indirectly supported by the data presented here.

5. CONCLUSIONS

From the results, the following conclusions can be drawn:

1. Aggregation of carrot cells in culture arises from an effect of pH on the cell wall rather than a selection of aggregating or disaggregating genotypes.

2. Electron micrographs show a lack of middle lamella and overall less wall in cells grown at pH 4.5 than cells grown at pH 5.5.

3. Three times more Ca^{2+} is bound to walls grown at pH 5.5 than at pH 4.5. Applying chelators to cells does not produce the same size class distribution pattern as changing the pH. Supplying elevated levels of Ca^{2+} does not cause cells to aggregate.

4. β-galactosidase activity is lower in cells grown at pH 4.5 vs. cells grown at pH 5.5 and not in agreement with the results of Wallner and Nevins.

5. Cell wall carbohydrate composition of each of two fractions from cells grown at pH 4.5 vs. cells grown at pH 5.5 is similar. A significantly higher amount of uronic acid is found in pH 4.5 media CWM. There is a higher percentage of uronic acid and pentose in the Media CWM fraction, independent of pH, compared with fractions which comprise the native cell wall.

6. Cells grown at pH 5.5 contain 26% more cell wall material than cells grown at pH 4.5.

7. The Media CWM fraction primarily consists of high molecular weight polymers.

8. All fractions of cell wall from tissue grown at pH 5.5 have more hydroxyproline than in tissue grown at pH 4.5.

These results suggest three hypotheses concerning the mechanism of pH induced cell aggregation/disaggregation in carrot cultures. The first is the inhibition of synthesis of wall material at pH 4.5, the second is the inhibition of incorporation of wall material into the wall, and the third is a combination of the two. Though the data presented here do not distinguish between these hypotheses, this investigation does provide a foundation from which further experimental tests of these hypotheses can be undertaken. Isolation and characterization of the pectic material from the media should provide specific answers as to what component(s) of the cell wall are initially being effected by pH. The isolation and analysis of the HPCGs in the cell wall and in the media, should show whether the HPCG was released as monomer, cross-linked, or complexed with polysaccharide. Radiolabeling cell wall components such as hydroxyproline and following their incorporation into the wall will provide information on the effect of pH on the rate of synthesis or incorporation of cell wall components. In addition, isodityrosine can be measured to determine whether the amount is different at the two pHs. These and other experiments will extend the information presented here and ultimately lead to an understanding of the mechanism of plant cell aggregation.

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