1973

Cell aggregate development in suspension cultures of Paul's Scarlet rose

Stephen John Wallner

Iowa State University

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Iowa State University, Ph.D., 1973
Botany

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Cell aggregate development in suspension cultures of Paul's Scarlet rose

by

Stephen John Wallner

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Botany and Plant Pathology
Major: Botany (Physiology)

Approved:

Signature was redacted for privacy.

In Charge of Major Work
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INTRODUCTION

Cultured plant cells offer the unique opportunity for detailed studies of plant biochemistry, physiology, and developmental morphology in the absence of complex tissue. However, the application of tissue culture techniques to problems in these various areas has not advanced the overall understanding of such phenomena as much as might have been predicted when tissue culture was introduced as a physiological tool. The difficulty appears to stem from the fact that cells in culture are subject to different constraints than the cells of intact tissues. As a result, interpretation of the physiological responses of cultured cells has been difficult and the extrapolation of data from one system to another is far from valid.

To make credible comparisons between intact and cultured tissue, the basic biological processes of cells in culture must be understood. It is therefore imperative that baseline studies on the behavior of cultured cells be conducted.

One of the most conspicuous aspects of cell growth in suspension culture is the tendency to produce cell aggregates of extremely variable dimensions. This heterogeneity of cell suspensions is perhaps the most imposing barrier to their more widespread and successful utilization.

One of the goals of this research was to describe the factors that regulate the size of cell aggregates. The
ultimate purpose was to examine the role of the cell wall in the surface reactions involved in aggregate development in suspension cultures of Paul's Scarlet rose tissue. In addition to its contribution to present knowledge of the higher plant cell surface, an understanding of how aggregates form and dissociate may lead to successful efforts to prevent their occurrence or limit their size. This would increase the value of suspension cultures as experimental tools.
Presumably, the interactions that regulate the formation and dissociation of cell aggregates in suspension-cultured plant tissue occur at the cell surface. However, very little is known about the topography of the plant cell surface and its significance in various aspects of plant development. Therefore, a brief review of the extensive literature dealing with the surface interactions of animal cells is appropriate here to provide a biological perspective for the consideration of the situation in cultured plant cells.

Cell surface interactions involved in cell aggregation and separation are of fundamental importance in a wide variety of biological systems. The surface of animal cells apparently participates in the developmental organization of cells into organs and tissues. Experiments with artificially dissociated cells of mammalian or avian embryos have shown that cells recombine under the influence of surface specificities that direct the formation of intercellular bonds (Moscona, 1963). Such surface interactions often appear to transcend species differences in the formation of bispecific tissues. Dissociated kidney, liver, retina, and other cells derived from mouse embryos will aggregate with the corresponding cell types from chicken embryos but not with cells from unlike mouse tissue (Moscona, 1961). The role of cell surface specificities in guiding normal animal morphogenesis is under intensive
Moscona (1963) proposed that the specificity of various cell associations may be due to an interaction between complementary macromolecules on the cell surfaces. Many studies have indicated that these macromolecules are glycoprotein in nature. The surface modifications that accompany the transformation of animal cells to the malignant state (Burger, 1971; Edwards, Campbell, and Williams, 1971) result in differences between the surface glycopeptides of control and virus transformed cells (Buck, Glick, and Warren, 1971). Phytoagglutinins, proteins or glycoproteins that bind carbohydrates, have been used to map the surface of various animal cells (Sharon and Lis, 1972). Agglutinin receptor sites, perhaps glycoprotein, may be permanently exposed on the surface of transformed cells (Burger, 1972). The species specific reaggregation of dissociated sponge cells also appears to be due to surface glycoproteins that occur in particulate units (MacLennan, 1963; Margoliash, et al., 1965). Moscona (1967) isolated a macromolecule from live sponge cells which specifically enhanced their aggregation and suggested that it consisted mostly of glycoprotein.

The features of the cell surface that form the basis of cell to cell recognition are of obvious importance in the sexual union of gametes. Specific differences in the oligosaccharides at the surface of rabbit and hamster spermatozoa have been demonstrated by Nicolson and Yanagimachi (1972).
Crandall and Brock (1968) isolated glycoproteins from the cell surfaces of *Hansenula wingei*, a sexually agglutinative yeast. They presented evidence that these glycoproteins may be responsible for the specificity of agglutination between opposite mating types. In chlamydomonas high molecular weight glycoproteins appear to be the complementary substances involved in the union of different mating types (Wiese, 1965).

Virus-cell interactions also depend on cell surface recognition. Losick and Robbins (1969) showed that phage recognition sites of the bacterial cell wall (O-antigen portion) are composed of polysaccharides.

The morphogenetic aggregation of amoeboid slime mold cells is another example in which specific cell surface interactions may be involved. Garrod (1972) has shown that the cohesive-ness of slime mold cells changes with various stages of its life cycle.

Morphological changes in *Neurospora crassa* occur in association with changes in cell wall structure (deTerra and Tatum, 1961). The addition of L-sorbose to the culture medium induces a colonial form of growth and furthermore, it appears that sorbose acts at the cell surface.

Indeed, even a cursory review of the literature confirms Kalckar's (1968) statement that the "molecular basis for cell surface patterns governing the 'social characteristics' of a cell has become a great chapter in general biology." This chapter, however, includes very little information regarding
higher plant cells. The possibility exists that the cell wall may harbor macromolecules which are similar, at least in general biological function, to those found at the surface of animal and lower plant cells. However, the relationship of the biochemical structure of the cell surface to its function in cellular recognition and interaction in higher plants has not been described in meaningful terms. This omission does not appear to be due to a lack of important surface reactions in plant biology since there are many examples of intercellular relationships that may involve the cell surface in some way.

Ontogenetic fusions are known in higher plants; these occur most commonly in the developing flower, and particularly in the gynoecium. In numerous apocarpous genera, it has long been known (Bessey, 1898) that the ovarian cavity is formed by a developmental fusion of primordial tissue. Baum (1948a) noted that the region of contact in this type of fusion is characterized by various degrees of interpenetration of the epidermal cells and that the cuticle over the area of contact is very thin. In certain flowers with syncarpous gynoecia the ovary develops by the fusion of carpel primordia which are separate in their early stages of development (Fahn, 1967).

Graft unions are established through a type of fusion. It may be that the cellular basis for graft incompatibility involves a cell surface reaction, although very little information is available on this subject. Buchloh (1960) has studied this problem and suggests that "the formation of strong grafts
could be intimately related to processes which unite the adjoining cells of stock and scion as soon as they have come into close contact. Weak union, on the other hand, results from the inhibition of one or more of these processes."

In the plant body gliding growth requires that the walls of neighboring cells be allowed to slide over one another. Intrusive growth, in which the growing cell penetrates between existing cells, involves the establishment of new areas of contact and attachment between cells (Fahn, 1967). It seems very likely that these types of growth are at least partially under the control of the plant cell surface.

Leaf abscission is another phenomenon in plant biology that involves the cell surface. In some species the molecular modifications of the wall that result in abscission are more subtle than a dissolution of intercellular material. In these species cell contacts at the separation layer are weakened although no apparent dissolution takes place (Fahn, 1967).

Other plant cell to cell interactions that may involve changing surface relationships include the sloughing of cells from the root cap, microspore formation, fruit ripening, the development of intercellular spaces, various host-pathogen situations, and pollen tube growth and incompatibility.

Tissue culture techniques have been used with great success in many of the studies that have contributed to the present knowledge of the animal cell surface. Similarly, plant tissue culture methodology may be useful in the investigation
of various aspects of the interactions of plant cell surfaces. A beginning in this direction was recorded by Gautheret (1945) who demonstrated that when tissue cultures isolated from willow and poplar were placed next to each other they fused following cell proliferation. However, contact between tissue of elder and maple did not result in fusion, and in fact, the cultures died several hours later. Duhamet (1957) also studied the growth of plant tissue cultures of different origin when placed one on top of the other. Surface interactions in mixed callus cultures have been studied perhaps most thoroughly by Ball (1969, 1971). He used cultures isolated from a large number of different angiosperms and obtained mixed calli by shaking two of the callus tissues in liquid medium before transfer to an agar-solidified medium. Various patterns of interaction were observed after one month of growth; certain combinations yielded tissue mixtures formed as the result of the union of adjacent cell walls. Earlier, Ball (1963) had observed that certain plant cells from suspension culture adhered to glass that had been cleaned by the method of Rappaport and Bishop (1960). He felt that this attachment of plant cells to a glass surface was comparable to that which is considered normal in cultures of animal cells. The attachment of animal cells to glass has been utilized to study the mechanism of cell adhesion (Weiss, 1964). Ball and Joshi (1965), studying tobacco cells in small glass observation chambers noted that only those that adhered to
the glass were seen to undergo cell division.

Liquid suspension cultures afford a convenient system for the study of the higher plant cell surface. Of special interest in this regard are the surface reactions that regulate the development of cell aggregates in plant suspension cultures. Suspension cultures typically contain cell aggregates of extremely variable dimensions. These aggregates are tissue masses that are usually composed of unspecialized, parenchymatous cells. The histology of cell aggregates has been described in detail by Halperin and Jensen (1967) with carrot and Sussex and Clutter (1967) with *Eucalyptus* suspension cultures. The fine structure of the external surface of cell aggregates and areas of cell contact within aggregates has been examined by Davey and Street (1971), Leppard, et al. (1971), and Leppard and Colvin (1971). As pointed out by Steward, Mapes, and Ammirato (1969) and Wareing and Phillips (1970) cell aggregates are formed from cells which remain attached after they divide; apparently plant cells do not come together into clusters in the way that some cultured animal cells do. Aggregate size, that is the number of cells in an aggregate, is a morphological characteristic of the tissue from which the culture is derived; it is influenced by the composition of the nutrient medium and culture age (Street and Henshaw, 1963).

Cell aggregates present a problem because they contain unknown gradients of diffusion and varying surface to volume
ratios. In addition, large aggregates prevent the accurate transfer of uniform cell suspensions as well as the culture of higher plant cells in chemostats.

Lamport (1964) classified a variety of suspension cultures on the basis of their pipettability which ranged from "nil" to "excellent". Cultures of *Acer pseudoplatanus*, *Phaseolus vulgaris*, and Paul's Scarlet rose showed a relatively high degree of cell separation; not surprisingly, these are three of the most commonly used plant cell suspensions.

The usefulness and potential of liquid shake cultures is evidenced by their utilization in many diverse investigations. These have included studies of cell division (Stuart and Street, 1969), transport across membranes (Maretzki and Thom, 1972), cell wall synthesis (Rubery and Northcote, 1970), the mechanism of hormonal action (Street, Collin, Short, and Simpkins, 1969), secondary metabolism (Hahlbrock, Kuhlen, and Lindl, 1971), and many others. Various advantages of plant suspension cultures for working at the cellular level were pointed out by Tulecke (1964), Venketeswaran and Chen (1964), and Street, et al., (1965). However, the full potential of the suspension culture technique has not been realized due to the inability to culture higher plant cells like microorganisms, that is, completely separated from one another. True single cell suspensions have not been obtained from any species (Henshaw, et al., 1966). A suspension containing a high proportion of single cells or very small cell aggregates
is due to a reduced tendency for dividing cells to stick together or to the fragmentation by cell separation of large, previously formed aggregates.

Since the introduction of present suspension culture techniques by Nickell (1956) patterns of growth and development have been described for numerous species. Suspension cultures are established by transferring callus tissue fragments to an agitated liquid medium of appropriate composition. The growth cycle of batch propagated suspension cultures is begun by transferring an aliquot of a cell suspension to fresh culture medium. In most cases there appears to be a critical minimum inoculum cell density below which growth in subculture will not occur (Street, et al., 1965).

Henshaw, et al. (1966) followed changes in total cell number in suspension cultures of *Parthenocissus tricuspidata* and *Acer pseudoplatanus* and were able to distinguish sequential stages of growth. A lag phase characterized by no detectable increase in cell number follows inoculation. The size of the inoculum, i.e. the initial cell density, influences the duration of the lag phase; reducing the initial density usually lengthens this phase of the culture cycle. Following the lag phase, Henshaw, et al. (1966) observed a phase of rapid cell division in which most of the cells participate; a phase of deceleration of cell division, during which average cell size increases and cell separation commences; and a stationary phase, during the early part of which cell size continues to
rise, cell separation reaches its maximum, and cell number remains constant. Similar growth patterns have been found in suspensions of *Phaseolus vulgaris* (Mehta, Henshaw, and Street, 1967; Liau and Boll, 1971); *Haplopappus gracilis*, *Convolvulus arvensis*, *Daucus carota* (Torrey, Reinert, and Merkel, 1962); *Rubus fruticosus* (Street and Henshaw, 1963); and *Linum usitatissimum* (Mehta, et al., 1967).

In most suspension cultures there is a tendency of the cell aggregates to progressively increase in size during the phase of rapid cell division (Street, et al., 1965). Mitotic figures are much more common in tissue pieces (large aggregates) than in single cells and small cell groups (Torrey, et al., 1962; Mehta, et al., 1967; Sussex and Clutter, 1967; Henshaw, et al., 1966). Cell separation leading to aggregate dissociation does not occur to a significant degree until mitotic activity has almost ceased (Torrey and Reinert, 1961; Torrey, et al., 1962; Henshaw, et al., 1966; Liau and Boll, 1971). Street and Henshaw (1966) felt that cell division and separation are "mutually antagonistic" processes.

Cell expansion, which also occurs primarily after the phase of most rapid cell division (Liau and Boll, 1971; Henshaw, et al., 1966) has been causally related to cell separation (Lamport, 1964; Henshaw, et al., 1966). Some of the earliest experimental studies with suspension cultures showed that the surface interactions that control the degree of cell separation are sensitive to changes in the
composition of the nutrient medium. Reinert (1956) found that deficiencies of folic acid and vitamin B₁₂ increased cell dispersion in suspension cultures of spruce tissue. Similarly, the omission of choline or ascorbic acid resulted in greater tissue dissociation in carrot suspensions (Torrey and Reinert, 1961). Venketeswaran and Chen (1964) observed that the presence of yeast extract increased the number of single cells and small cell aggregates in suspensions derived from Euphorbia and Lactuca. In liquid shake cultures of pea root callus, separation was promoted by an appropriate balance between auxin and yeast extract (Torrey and Shigemura, 1957); a proper ratio of auxin and coconut milk produced a similar response in sycamore cell suspensions (Lamport and Northcote, 1960). The above observations may, in part, reflect the specific responses of different kinds of tissue, but no general pattern is apparent from the results of these studies.

The most consistently reported effect of medium composition on aggregate formation is that due to increased auxin concentration. Bergmann (1960) found that 2,4-D increased the proportion of free cells in liquid cultures of Nicotiana and Phaseolus. Torrey and Reinert (1961) reported that the absence of 2,4-D greatly reduced the production of free cells in suspensions of carrot and Convolvulus tissue. Lamport (1964) noted that increased levels of 2,4-D enhanced the "pipettability" of sycamore cell suspensions. Halperin and Jensen (1967) found that the presence of 2,4-D resulted in the
fragmentation of carrot cell clumps into smaller aggregates. Tissue friability and the abundance of free cells were increased by naphthaleneacetic acid in suspension cultures of sunflower crown gall tissue (Hayashi, 1965). A similar effect of NAA was noted by Hart, Woodcock, and Wilson (1970) in liquid cultures of *Pogostemon cablin*. Digby and Wareing (1966) found that sycamore cells tended to be more loosely aggregated in the presence of indoleacetic acid.

The promotive effect of auxin on cell dispersion in plant suspension cultures is not a universal one, however. Smaller aggregates were formed without 2,4-D in *Digitalis* suspension cultures (Staba and Lamba, 1963). Similar results were obtained by Ranganathan, et al. (1962) who worked with *Vigna catjang* tissue. Likewise, Pillai and Hildebrandt (1969) reported that low concentrations of 2,4-D favored the dissociation of geranium callus in liquid culture.

On solid media, it has been found that a high auxin concentration relative to kinetin promotes tobacco callus growth that is loose and watery (Skoog and Miller, 1957; Feng and Linck, 1970).

Kinetin was found to decrease cell separation in *Haplopappus* suspensions (Eriksson, 1965). Sycamore cells also showed an increased tendency to adhere at high kinetin concentrations (Simpkins, Collin, and Street, 1970).

It is important to point out that the influence of medium composition on aggregate development has not been
assessed under well described cultural conditions. For example, the effect of auxin on cell aggregation has not been discussed in relation to the specific stage of culture development. As a result, interactions with other factors, particularly culture age, may be responsible for the contradictory reports concerning medium effects.

There is little information available that indicates how various nutritional factors enhance or suppress the dissociation of cell aggregates in suspension culture. However, any explanation of aggregate formation and dissociation must directly or indirectly involve the physiology and biochemistry of the cell surface. Lamport (1964) recognized this and called the primary cell wall "part instrument" of morphogenesis and further states that, regardless of the chemical basis for cell adhesion, the separation of cells is favored "by conditions which minimize the mechanical stabilization of adjacent walls."

In studies of the origin of single cells in suspension cultures, Sussex (1965) suggests that attention be given to the physiology of the wall. Street, et al. (1965) indicate that to culture higher plant cells like microorganisms implies the ability to block or modify the production of "cementing substances" which normally occur between them. Advances in the biochemistry of wall formation and composition are needed before its hormonal regulation can be used to produce true free cell cultures (Street and Henshaw, 1963; Street, et al., 1965).
Torrey and Reinert (1961) and Halperin and Jensen (1967) feel that auxin-induced dissociation of cell aggregates is the result of auxin-induced changes in the cell wall. Fuller and Grant (1968) found a difference in cell wall composition between a friable and non-friable strain of *Vicia faba* callus grown on semi-solid media.

The detailed descriptions of aggregate dissociation given by Halperin and Jensen (1967) and Sussex and Clutter (1967) suggest that it may be an enzyme-mediated process. Sussex (1965) mentioned the possibility that cultured cells may produce substances which cause the separation of adherent cells. More recently, Liau and Boll (1971) suggested that cell separation in suspension cultures of *Phaseolus vulgaris* might follow the synthesis of a hypothetical extracellular enzyme which solubilizes the material holding cells together. Enzymes capable of attacking chemical bonds between cell wall components are of particular interest in this regard. It is well established that polysaccharide-degrading enzymes are associated with the cell wall in intact plant systems (Heyn, 1969; Nakagawa, et al., 1971; Nevins, 1970).

Mandels, Parrish, and Reese (1967) found β-1,3 glucanase activity in homogenates of agar-grown callus tissue derived from bean, lettuce, carrot, and pepper. Gamborg and Eveleigh (1968) measured the activity of intracellular enzymes isolated from suspension cultured cells and extracellular enzymes.
obtained from the culture media. The enzyme preparations were active against a variety of polysaccharide substrates. The culture media of wheat and barley suspensions contained activity towards laminarin, pustulan, cellobiose, and maltose; the greatest of these was laminarinase. Cultures of rose, horseradish, and soybean contained laminarinase, but it was low in comparison to that of the cereal cultures. Hughes (1968) studied β-glucosidase activity in homogenates of white clover callus tissue grown in liquid culture; the substrate used was p-nitrophenyl-β-D-glucoside. Keegstra and Albersheim (1970) found glycosidases located at the surface of suspension cultured sycamore cells. They showed that the surface enzyme preparation catalyzed the release of reducing sugars from isolated cell wall material. Klis (1971) reported that α-glucosidase activity could be extracted from the surface of living cells of Convolvulus arvensis callus tissue.

Street, Mansfield, and King (1971) were able to maintain a high degree of cell separation in sycamore cultures by incorporating low concentrations of pectinase (Macerozyme) and cellulase (Onozuka P5100) into the medium. However, the addition of these crude enzyme preparations to culture media introduces new and undefined variables. Furthermore, plant cells and cell walls vary greatly in their sensitivity to enzyme mixtures of this type (Otsuki and Takebe, 1969). Although the presence of the enzymes did not reduce cell growth in the sycamore suspensions used by Street, et al. (1971) it seems unlikely
that this would hold true for most other tissues. This type of approach is rather negative; it is not directed at the developmental events that regulate cell aggregation in culture. The degree of cell dispersion in suspension cultures will be subject to precise experimental control only when the underlying mechanisms of aggregate formation and dissociation are well understood.
MATERIALS AND METHODS

Plant tissue and cultural conditions

The tissue used in this study was derived from that which was originally isolated in 1957 from sections of young stem material of Paul's Scarlet rose (Rosa sp.) (Nickell and Tulecke, 1959).

Table 1 lists the components of the standard medium used throughout this study; it is a modification of the PN-25 medium described by Tulecke, Taggart, and Colavito (1965). All components of this and the various experimental media were autoclaved together.

Stock callus cultures were maintained on standard medium solidified with 0.75 per cent agar and were subcultured every 30 days. They were kept in darkness at room temperature. Cultured under these conditions, the tissue is parenchymatous; it contains no recognizable differentiated cells. Cell suspensions were initiated by transferring 1.5 to 2.0 g of callus tissue (3 to 4 weeks old) to 500 ml Erlenmeyer flasks containing 125 ml of liquid standard medium. After 9 or 10 days, 10 ml of the resulting suspension was transferred to 125 ml of fresh liquid medium; subsequent transfers were on a regular basis at intervals of 13 days. All cell suspensions were incubated at 26 °C in the dark on shakers (New Brunswick Co.) operated at a rate of 100 excursions per minute.
Table 1. Composition of standard medium\(^a\) used in tissue culture studies of Paul's Scarlet rose

<table>
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<th>Component</th>
<th>mg/liter</th>
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<th>mg/liter</th>
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<tr>
<td>Ca(NO(_3))(_2) (\cdot) H(_2)O</td>
<td>280.0</td>
<td>CoCl(_2) (\cdot) 6H(_2)O</td>
<td>0.01</td>
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<tr>
<td>NaNO(_3)</td>
<td>1800.0</td>
<td>CuSO(_4) (\cdot) H(_2)O</td>
<td>0.02</td>
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<tr>
<td>KNO(_3)</td>
<td>80.0</td>
<td>H(_3)BO(_3)</td>
<td>0.2</td>
</tr>
<tr>
<td>KCl</td>
<td>900.0</td>
<td>KI</td>
<td>0.5</td>
</tr>
<tr>
<td>NaH(_2)PO(_4) (\cdot) H(_2)O</td>
<td>300.0</td>
<td>MnSO(_4) (\cdot) H(_2)O</td>
<td>0.8</td>
</tr>
<tr>
<td>MgSO(_4) (\cdot) 7H(_2)O</td>
<td>760.0</td>
<td>Molybdic acid</td>
<td>0.01</td>
</tr>
<tr>
<td>Na(_2)SO(_4)</td>
<td>200.0</td>
<td>ZnSO(_4) (\cdot) 7H(_2)O</td>
<td>0.50</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>5.0</td>
<td>Adenine</td>
<td>10.0</td>
</tr>
<tr>
<td>Ca pantothenate</td>
<td>1.0</td>
<td>Inositol</td>
<td>100.0</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>1.0</td>
<td>Sorbitol</td>
<td>100.0</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
<td>Naphthaleneacetic acid</td>
<td>1.0</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>0.5</td>
<td>Kinetin</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glucose</td>
<td>20,000.0</td>
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\(^a\) pH was adjusted to 5.5 with NaOH.
In each experiment cells were cultured in 125 ml Erlenmeyer flasks containing 30 ml of medium. Experimental media were inoculated with cells from 13-day-old stock suspension cultures which were in passage 2, 3, or 4. Inoculum suspensions were prepared by pouring off the spent medium and re-suspending the cells in 250 ml of fresh medium; this was done to minimize the carry-over of components of the conditioned medium. Three ml of this suspension was used to inoculate each flask. This resulted in an initial cell density of 4000 to 5000 cells per ml. There were three replicate cultures per treatment.

Growth measurements

In most studies involving the growth of plant cells in suspension, aggregate development has been only qualitatively observed and described. However, in some cases, various sieving methods have been used to obtain a relative, semi-quantitative measure of the degree of cell aggregation (Torrey and Reinert, 1961; Eriksson, 1965; Sussex and Clutter, 1967; Rajasekhar, et al., 1971). These methods are based upon the isolation of cell aggregates of various sizes determined by the pore size of the sieves through which the suspension is passed. The contribution of one or more of the size classes to the total culture weight is used to estimate the degree of cell aggregation or separation. One problem inherent in these measurements is that they do not distinguish between the
contributions of cell size and cell number to aggregate size. Suspension cultures typically contain cells that vary greatly in size. Average cell size is known to change with culture age (Henshaw, et al., 1966; Liau and Boll, 1971) and with changes in composition of the culture medium (Digby and Wareing, 1966; Street, et al., 1969). Microscopic examination of the rose cultures used in this study revealed that aggregates of approximately equal size often contain unequal numbers of cells (Figure 1). This disadvantage associated with sieving methods has been noted by Torrey, et al. (1962), and Sussex (1965).

A more meaningful expression of the degree of cell aggregate formation is the average number of cells per aggregate. This was obtained by counting the number of aggregates and the total number of cells present in aliquots removed from the same cell suspension. The three culture flasks of each treatment were usually combined and diluted to facilitate counting. The number of aggregates per ml was determined with a Sedgewick-Rafter Counting Cell (A. H. Thomas Co.). Single cells were counted as one aggregate. To obtain accurate cell counts it was necessary to first disperse the cell aggregates. Therefore, an aliquot of the suspension was treated with a solution of 0.2 per cent Pectinase (w:v) (Sigma Chem. Co.) in 50 mM sodium citrate buffer (pH 4.6) for 1 to 2 hr followed by vigorous agitation with a vortex mixer for 30 seconds. This treatment was found to produce near complete dispersion of rose cell
Figure 1. Aggregates of similar size that contain widely different numbers of cells. (x85).
aggregates (Figure 2). The activity of Sigma pectinase against various substrates is shown in Table 2. Other workers (Henshaw, et al., 1966; Liau and Boll, 1971) have used prolonged (12 to 16 hr) incubation with either pectinase or chromic acid solutions to obtain separated cells but such treatment of rose tissue resulted in extensive cell disintegration. After appropriate dilution, cells were counted with the Sedgewick-Rafter cell. The average number of cells per aggregate was calculated from the aggregate and cell counts. Each count was the average of ten microscope fields in each of four 1 ml aliquots.

An estimate of cell size was obtained by centrifuging a sample of the suspension for 5 minutes (ca. 2200 g) in a graduated centrifuge tube and calculating the packed cell volume (PCV) per $10^6$ cells.

Dry weight was determined by freeze-drying cells after separating them from the medium by sieving through Miracloth (Calbiochem). Miracloth retains cells and aggregates of all sizes.

Cell wall preparation

Cells were removed from culture media by filtration through Miracloth, rinsed thoroughly with distilled water, and freeze-dried. Freeze-dried cells were suspended in 20 ml of cold 0.02 M potassium phosphate buffer (pH 6.9). The suspended cells were broken in a French pressure cell under a pressure
Figure 2. Time course of aggregate dispersion in 0.2 per cent Sigma pectinase.
Table 2. Relative activity of Sigma pectinase against various substrates

<table>
<thead>
<tr>
<th>Model substrates</th>
<th>Relative Activity</th>
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<tbody>
<tr>
<td>p-nitrophenyl-β-D-glucoside</td>
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<tr>
<td>p-nitrophenyl-β-D-galactoside</td>
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</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>p-nitrophenyl-α-D-galactoside</td>
<td>58</td>
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<tr>
<td>p-nitrophenyl-α-D-xyloside</td>
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<table>
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<th>Polysaccharides</th>
<th>Relative Activity</th>
</tr>
</thead>
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<tr>
<td>polygalacturonic acid&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
<td>pectin&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70</td>
</tr>
<tr>
<td>carboxymethylcellulose&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>laminarin&lt;sup&gt;c&lt;/sup&gt;</td>
<td>46</td>
</tr>
<tr>
<td>araban&lt;sup&gt;d&lt;/sup&gt;</td>
<td>31</td>
</tr>
<tr>
<td>xylan&lt;sup&gt;d&lt;/sup&gt;</td>
<td>46</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sunkist Co.
<sup>b</sup>Hercules, Inc.
<sup>c</sup>Pierce Chem. Co.
<sup>d</sup>Koch-Light Laboratories.
of approximately 358 Kg per cm². After two passages through the pressure cell the phosphate buffer was removed by filtration through Miracloth, the cell walls resuspended in 20 ml of fresh buffer, and passed through the French pressure cell twice more to ensure complete breakage. The buffer was again removed by filtration. The crude wall preparation was then transferred to a sintered glass funnel (medium porosity) and suspended in 20 ml of acetone. After 10 minutes the acetone was removed and the walls rinsed twice with 10 ml of acetone. Walls were then suspended in 20 ml of chloroform:methanol (1:1, v:v) for 10 minutes after which it was removed and the walls rinsed twice with 10 ml of the same solvent mixture. The cell walls were then air-dried at room temperature and placed in a desiccator with phosphorus pentoxide for at least 48 hr in vacuo.

Wall hydrolysis and sample preparation

The composition of the non-cellulosic polysaccharide fraction of the isolated cell walls was determined using a method described in detail by Jones and Albersheim (1972) and Loescher (1971). Figure 3 summarizes the steps in this procedure. Component sugars were obtained from 6 to 8 mg of dried wall material by an initial 1 hr hydrolysis at 121°C with 2.0 ml of 0.2 N trifluoracetic acid (TFA) and subsequent enzymolysis for 6 hr with a mixture of polysaccharide-degrading enzymes produced by the fungus Sclerotium rolfsii. The TFA
Figure 3. Flow sheet diagram of the procedure used to analyze the non-cellulosic fraction of wall samples.
solution contained inositol (0.5 mg/ml) as an internal standard.

A culture of *S. rolfsii* (Isolate 14) was obtained from Dr. D. F. Bateman. The fungus was grown at room temperature for 10 days on autoclaved 8-day bean (*Phaseolus vulgaris* var. Red Kidney) hypocotyls. Enzyme preparation was similar to the method of VanEtten and Bateman (1969).

Several experiments were conducted to determine whether the hydrolytic procedure described by Jones and Albersheim (1972) was suitable for the analysis of walls isolated from cultured rose cells. The first two of these were designed to compare the effectiveness of various concentrations of TFA in releasing component sugars from rose cell walls.

The interaction of temperature and acid concentration was determined by comparing hydrolysis with 0, 0.02, 0.20, and 2.0 N TFA at 25 and 121 C. As shown in Figure 4, subsequent enzyme hydrolysis is most effective with a 0.2 N TFA pretreatment at 121 C. None of the other TFA-temperature combinations produce a satisfactory yield of all sugars. Xylose linkages appear to be particularly resistant to both TFA and enzymatic hydrolysis.

The relative effectiveness of TFA hydrolysis not followed by enzymolysis is shown in Figure 5. Wall samples were treated for 1 hr at 121 C with 0, 0.02, 0.2, or 2.0 N TFA; this was followed by either a 6 hr incubation with enzyme or by incubation in buffer. The most effective of these hydrolysis
Figure 4. The interaction of acid concentration and temperature during TFA pretreatment on the release of monosaccharides by *S. rolfsii* enzymes. Solid circles = 121°C; open circles = 25°C.
Figure 5. The effect of pretreatment with various concentrations of TFA on the release of monosaccharides after a 6 hr incubation in *S. rolfsii* enzymes (solid circles) or buffer (open circles).
The diagram shows the relationship between TFA Concentration (N) and the mg sugar per 100 mg wall for different sugars:

- **rham**
- **glu**
- **ara**
- **xyl**
- **gal**
- **galur**

As the TFA Concentration increases, the mg sugar per 100 mg wall also increases for most sugars, with specific trends observed for each.
combinations was 0.2 N TFA followed by enzyme treatment (Figure 5). Arabinose is readily solubilized by all acid concentrations (Figure 5a); the enzyme mixture does not further increase arabinose yield from walls exposed to 0.02 N TFA. None of the other wall components are effectively released by 0.02 N TFA alone. Arabinose, galactose, and galacturonic acid are liberated in substantial amounts by enzyme treatment alone, i.e. with no TFA pretreatment. The yields of glucose and especially of galacturonic acid are enhanced by enzyme treatment after initial hydrolysis in all TFA concentrations. Xylose yield is not increased by the S. rolfsii enzyme mixture after any TFA pretreatment.

The effectiveness of the 6 hour enzyme incubation period was also tested. Enzymatic hydrolysis of rose cell walls pretreated with 0.2 N TFA was followed over a 12 hr time course. As shown in Figure 6, maximum yield of component sugars is obtained by 6 hours.

The results represented in Figures 4, 5, and 6 provide ample evidence that the conditions described by Jones and Albersheim (1972) are optimum for the hydrolysis of walls isolated from cultured rose cells.

Cell wall-degrading enzymes produced by S. rolfsii are induced on a substrate of bean cell walls (hypocotyls). An important question may be raised that growth on a substrate induces specific enzymes that are particularly effective in its hydrolysis. To test this possibility, S. rolfsii was
Figure 6. Time course of the release of monosaccharides from rose cell walls by *S. rolfsii* enzymes after 0.2 N TFA pretreatment.
grown on 100 g of autoclaved rose cells harvested from liquid culture. The fungus showed a longer lag phase on this substrate, but after 10 days its growth was near that obtained on bean hypocotyls. The enzyme mixture was prepared as described previously and compared to that from bean-grown *S. rolfsii* cultures. However, *S. rolfsii* grown on bean hypocotyls produces an enzyme mixture that is far more effective in releasing galacturonic acid from both bean and rose cell walls (Figure 7).

Following hydrolysis, the hemiacetal groups of the liberated monosaccharides were reduced by adding 0.5 ml of 1.0 N ammonium hydroxide containing 3.0 mg sodium borohydride and 1.0 mg of L-mannonic acid to each sample. The L-mannonic acid was used as an internal standard for the determination of the uronic acids in the sample; it was prepared as described by Jones and Albersheim (1972). After 1 hr at room temperature the reduction reaction was stopped and excess borohydride decomposed by adding glacial acetic acid.

The alditols and aldonic acids present in each sample after the borohydride treatment were separated by using Dowex 1-X4 anion exchange resin, 20 to 50 mesh, acetate form. The alditols present in the supernatant fluid after ion exchange constituted the non-cellulosic neutral fraction of the wall. After the resin pellets were washed twice with 5.0 ml of distilled water to remove any trapped alditols, the aldonic acids, comprising the uronic acid fraction, were removed from
Figure 7. Release of galacturonic acid from rose (shaded bars) and bean (open bars) cell walls by enzymes produced by S. rolfsii grown on either bean hypocotyls (B) or rose cells (R). A = no TFA pretreatment; B = 0.2 N TFA pretreatment.
mg galacturonic acid per 100 mg wall

B

R

B

R

A

B

R

0 0 0
the resin with 1.0 N hydrochloric acid. The aldonic acids were converted to aldonolactones by evaporating the acidic solutions to dryness at 50 C in a stream of filtered air. The aldonolactones were reduced to the corresponding alditols in the manner detailed by Jones and Albersheim (1972).

**Gas chromatography**

Using the technique of Nevins, et al. (1967), the alditols from the neutral sugar fraction and from the uronic acid fraction were converted to volatile acetate esters for analysis by gas chromatography. This involved reacting the alditols with 1 ml of acetic anhydride for 3 hr at 121 C.

Alditol acetate solutions were transferred to 2 ml serum vials; the acetic anhydride was removed by evaporation with a stream of filtered air. The residue was redissolved in 0.5 to 1.0 ml of methylene dichloride which reduces tailing of the solvent peak (Crowell and Barnett, 1967) and appears to prolong column life (Loescher, 1971).

Chromatographic conditions for the separation of alditol acetates were similar to those of Nevins, et al. (1967). Column material consisted of 0.2 per cent polyethylene glycol adipate, 0.2 per cent polyethylene glycol succinate, and 0.4 per cent XF-1150 silicone oil coated on Gas-Chrom P (100 to 200 mesh). All components of the column material were obtained from Applied Science Inc. About 1.2 g of column material was packed (with vibration) into 4 feet of washed 1/8 inch O. D.
copper tubing, 0.030 inch wall. Columns were conditioned at 180°C for 2 to 4 hr.

Chromatography was performed with a Packard 7311 dual column instrument equipped with flame ionization detectors. Helium was used as the carrier gas; hydrogen and compressed air were used for detector operation. Output was recorded on a Beckman 10-inch recorder.

Temperature programing was used to separate the neutral sugars; a Model 847 analog type programmer allowed non-linear programing of column oven temperature (Loescher, 1971). Starting temperature was 147°C; final temperature was 178°C. Injection port temperature was 200°C; detector temperature was 225°C. Separation of the uronic acid samples did not require temperature programing; oven temperature was maintained at 178°C. All other conditions were the same.

Three replicate injections were made of each sample. Peak heights and widths were measured. Using an Omnitab computer program, the peak areas were determined by triangulation and converted to mg of each component per sample; this was expressed as the mg of sugar per 100 mg of starting wall material. Standards of all sugars were run with each set of samples.

**Cellulose estimation**

The insoluble residue (2 to 3 mg) remaining after hydrolysis of cell walls with 0.2 N TFA and the *S. rolfii*
enzymes was subjected to 1.0 ml of 72 per cent (w:w) sulfuric acid for 1 hr at 30 C with vigorous agitation at 5 minute intervals. It was then diluted to a total volume of 29 ml and subjected to an additional hour at 121 C. An estimate of the cellulose content of the cell walls was obtained by means of an anthrone determination (Spiro, 1966) for total carbohydrates in the sulfuric acid hydrolysate of the wall residue. Glucose was used in preparation of a standard curve.

Enzyme preparation and assays

Cells grown in suspension were separated from the culture medium by filtration through Miracloth. The culture medium was centrifuged at 26,000 g for 30 minutes at 2 C to remove insoluble material. The clarified cell-free medium was then dialyzed for 36 hr against several changes of cold distilled water and used directly in the enzyme assays.

The cells removed from suspension were washed thoroughly with distilled water to remove any residual culture medium. Cell surface enzymes were extracted by soaking the live, unbroken cells in cold 0.5 M sodium citrate buffer (pH 4.6) for 20 minutes. Cells were removed from the buffer solution by filtration through Miracloth. The cell surface enzyme preparation was then dialyzed for at least 36 hr against several changes of cold distilled water.

Glycosidase activity of the enzyme preparations was detected by measuring the release of p-nitrophenol from glycoside
model substrates or reducing groups from polysaccharides. The various p-nitrophenyl glycosides were obtained from Pierce Chemical Co. or Koch-Light Laboratories. In all assays the glycosides were dissolved in 10 mM sodium citrate buffer (pH 4.6) at a concentration of 25 mM. Typical reaction mixtures contained 0.2 ml of substrate solution, 0.5 ml of 10 mM sodium citrate buffer (pH 4.6), and 0.3 ml of enzyme solution. The reactions were incubated at 30°C in a shaking water bath. Reactions were terminated by adding 2.0 ml of 200 mM sodium carbonate to each assay mixture. The concentration of liberated p-nitrophenol was determined by measuring optical density at 400 nm with a Beckman DB-G spectrophotometer; p-nitrophenol (Eastman) was used as a standard. Boiled enzyme controls were used in all assays.

To determine activity against polysaccharide substrates and isolated cell wall material, enzyme preparations were concentrated by lyophilization. The dried preparations were dissolved in 10 mM sodium citrate (pH 4.6). This treatment did not affect cell surface glycosidase activity; however, β-galactosidase activity of the culture medium was reduced 25 per cent after freeze-drying. Assays with polymeric substrates were performed by incubating 0.5 ml of concentrated (90 to 110 μg protein per ml) enzyme solution with 0.5 ml of substrate (5 mg per ml) at 30°C. The release of reducing sugars was determined by the dinitrosalicylic acid method (Lucksinger and Cornesky, 1962). This involved adding 1 ml of the color.
reagent to each reaction tube at the end of the incubation period and boiling them for 5 minutes. Absorbancy was measured at 540 nm; glucose was used as a standard. Boiled enzyme and substrate incubated in the absence of enzyme served as controls.

In some experiments isolated cell walls served as the substrate. Reaction mixtures contained 10 mg of wall material and 2.0 ml of concentrated (90 to 110 µg per ml) enzyme solution. Incubation time at 30 °C was 18 to 20 hours. Insoluble cell wall material was removed by centrifugation and washed twice with 70 per cent ethanol. The supernatant and ethanol washings were combined and evaporated to dryness at 50 °C with a stream of filtered air. Reducing groups released from the cell walls were detected with the dinitrosalicylate method.

The results of all enzyme assays were expressed as specific activity, i.e. µmoles product formed per hr per mg protein. Protein was determined by the method of Lowry, et al. (1951) using bovine serum albumin as a standard.
RESULTS

Most of this investigation was conducted in three separate but closely related studies. Initially, it was necessary to describe the pattern of growth, especially aggregate development, in cell suspensions. Also, to examine the mechanisms of cell adhesion and separation, it must be demonstrated that these processes are subject to some degree of regulation. Therefore, one objective of this study was to learn how to modify the composition of the culture medium in a way that affects aggregate size.

Information regarding the normal pattern of aggregate development and experimentally induced changes in this pattern made it possible to look for correlated changes in the biochemistry of the cell surface, i.e. the cell wall. Thus, in the second phase of this investigation, changes in cell wall composition were followed under conditions which were found to affect aggregate development. There may be specific cell wall modifications that influence the ability of the cell to participate in the surface reactions involved in aggregate formation and dissociation.

The third study was undertaken to explore the possibility that cell surface enzymes might play an important role in aggregate development. The emphasis here was on surface enzyme activity that would appear capable of bringing about cell wall changes.
Suspension culture growth and development

The batch propagation of plant cell suspensions is characterized by changing growth patterns in the cultured tissue (Henshaw, et al., 1966). Presumably, the populations of cells during the various stages of growth are not alike and may respond differently to experimental treatments. Therefore, the study of any aspect of suspension culture development requires a thorough description of the various patterns of growth during the culture cycle. The inoculation procedure used throughout this investigation produced an initial cell density of 4000 to 5000 cells per ml. This relatively light inoculum was used since it has been reported (Street, et al., 1971) that cultures initiated at low cell density show a longer and more clearly defined phase of most rapid growth and development thereby facilitating the study of culture variables that control this development. Changes in dry weight, PCV, and cell number in suspensions of Paul's Scarlet rose tissue during a typical 18-day culture period in the standard medium are illustrated in Figure 8. Following a lag phase of 5 days, cell number increases sharply until day 10; cell division activity is completed by day 12. Cell division and cell enlargement stages appear to be at least partially separated in time, since PCV increases most rapidly between 10 and 12 days and continues to increase after 12 days in culture, the time at which maximum cell number is reached. Dry weight accumulation is completed by day 15.
Figure 8. Dry weight, PCV, and cell number in rose cell suspensions during an 18-day culture cycle.
Changes in cell number, aggregate number, the average number of cells per aggregate, and cell size (PCV/10^6 cells) during an 18-day culture cycle are shown in Figures 9 and 10. The early stages are characterized by decreasing cell size and the formation of large cell aggregates. Cell aggregation reaches a maximum and cell size a minimum by day 8 (Figures 9, 10, and 11). At about day 10 to 12 cell separation and enlargement become the most conspicuous features of the cell suspension (Figure 9, 10, 13, and 14).

The increase in cell number during the first 8 days is not accompanied by a comparable increase in the number of aggregates formed, indicating that most of the cells produced during this period do not separate from one another following division. Between 8 and 12 days a large increase in the number of aggregates per ml results in a rapid decrease in the average number of cells per aggregate. After 12 days in culture, cell division has essentially ceased, but the increase in aggregate number continues at a high rate, indicating that the decrease in cells per aggregate after day 12 is due to the fragmentation of aggregates formed during the phase of rapid cell division.

Figures 9 and 10 represent averages of at least three experiments. However, it is somewhat misleading to state that aggregates are of a particular size at any one time. For, despite the fact that average aggregate size varies little between experiments and does change in a predictable manner,
Figure 9. Cell number, aggregate number, and aggregate size in rose cell suspensions during an 18-day culture cycle.
Figure 10. Cell size (diamonds) and aggregate size (circles) in rose cell suspensions during an 18-day culture cycle.
aggregates of many different sizes are always present.

Cells produced during the period of most rapid division occur mostly in large compact aggregates (Figure 11). By the end of the culture cycle, however, very few large aggregates remain; the degree of cell separation is high and average cell size is considerably greater (Figure 12). In most cases, cell separation and consequent aggregate dissociation are accompanied by cell enlargement (Figure 13). However, some aggregates were observed to loosen, develop intercellular spaces, and dissociate without extensive, concomitant cell expansion (Figure 14).

Enlarged cells most often occur at the periphery of cell aggregates; in loose aggregates that contain intercellular spaces; or as filamentous outgrowths of elongated cells. Free cells are usually much larger than cells of the large compact aggregates. Cell enlargement leads to a variety of cell shapes. Filaments or chains of elongated cells (Figure 15) are commonly formed; occasionally, these occur in spiral or helical form (Figure 16). Enlarged cells show particularly prominent cytoplasmic strands (Figure 17) and active protoplasmic streaming, readily visible with phase contrast microscopy.

Influence of culture medium

Aggregate size in many types of cell cultures is sensitive to changes in the medium. The manipulation of culture media
Figure 11. Cell aggregates typical of rose suspension cultures during the period of rapid division. (x85).

Figure 12. Appearance of rose suspension cultures during the later stages of the culture cycle. (x85).
Figure 13. Aggregate dissociation accompanied by extensive cell enlargement. (x85).

Figure 14. Aggregate dissociation not accompanied by extensive cell enlargement. (x85).
Figure 15. Filaments that form as the result of cell elongation and aggregate dissociation. (x85).

Figure 16. Coiled filament of elongated cells as observed under phase contrast. (x210).
Figure 17. Expanded cells in rose suspension cultures as observed with phase contrast microscopy.

a  x210.
b, c, d  x525.
has been the usual approach in attempts to obtain plant suspension cultures showing a high degree of cell separation.

Numerous sugars were tested initially for the ability to support growth of Paul's Scarlet rose tissue in suspension culture. As shown in Table 3, most of these were totally unsatisfactory substitutes for glucose. To further study the effect of sugar on tissue growth, pieces of rose callus from stock cultures (grown on agar-solidified medium) were transferred to solid media containing 2 per cent galactose, cellobiose, sucrose, or raffinose instead of glucose. After one passage, the tissue assumed a characteristic culture morphology on each carbon source. These cultures were maintained on solid media containing the respective carbon source by subculturing at 30 to 35 day intervals. The relative degree of callus growth on the various sugars changed noticeably with increased time in subculture (Table 4). Growth was very slight on galactose medium during the first several passages. However, during the next 24 months, the rose tissue apparently adapted to galactose as a carbon source since it now supports considerably greater callus growth. Conversely, the relative growth rate on raffinose decreased during this same period.

Sucrose, raffinose, galactose, and cellobiose were used to study the effect of the carbon source on aggregate size in liquid suspension cultures. The presence of galactose, raffinose, or sucrose in the medium results in the formation of the largest cell aggregates (Table 5). However, galactose,
Table 3. Growth$^a$ as affected by carbon source

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Dry weight increase per culture (mg)</th>
</tr>
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<tbody>
<tr>
<td>glucose$^c$</td>
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<tr>
<td>galactose</td>
<td>34</td>
</tr>
<tr>
<td>mannose</td>
<td>0</td>
</tr>
<tr>
<td>fructose</td>
<td>0</td>
</tr>
<tr>
<td>arabinose</td>
<td>0</td>
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<tr>
<td>xylose</td>
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</tr>
<tr>
<td>ribose</td>
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</tr>
<tr>
<td>rhamnose</td>
<td>0</td>
</tr>
<tr>
<td>sorbose</td>
<td>0</td>
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<tr>
<td>sucrose</td>
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<tr>
<td>melibiose</td>
<td>8</td>
</tr>
<tr>
<td>raffinose</td>
<td>104</td>
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</tbody>
</table>

$^a$Measurements taken after 15 days growth.

$^b$Each sugar was used at 20 g/l in the basal medium.

$^c$Control.
Table 4. Callus growth\textsuperscript{a} on various carbon sources as affected by time in subculture

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Relative callus growth\textsuperscript{b}</th>
<th>Passage 3\textsuperscript{c}</th>
<th>Passage 22\textsuperscript{c}</th>
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<td>glucose</td>
<td></td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>galactose</td>
<td></td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>cellobiose</td>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>sucrose</td>
<td></td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>raffinose</td>
<td></td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

\textsuperscript{a}After approximately 30 days.

\textsuperscript{b}A larger number indicates greater relative callus growth; an increment of 1 indicates a detectable difference in callus size upon visual comparison.

\textsuperscript{c}Each passage lasted 30 to 35 days.

Table 5. Growth and aggregate size\textsuperscript{a} as affected by carbon source

<table>
<thead>
<tr>
<th>Carbon\textsuperscript{b} source</th>
<th>Dry wt. per culture (mg)</th>
<th>Cell number per ml (X 10^{-3})</th>
<th>Aggregate size (cells per aggregate)</th>
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<tr>
<td>glucose</td>
<td>261</td>
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<td>100</td>
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<td>galactose</td>
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<tr>
<td>cellobiose</td>
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<td>138</td>
</tr>
<tr>
<td>sucrose</td>
<td>240</td>
<td>89</td>
<td>191</td>
</tr>
<tr>
<td>raffinose</td>
<td>92</td>
<td>52</td>
<td>247</td>
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</table>

\textsuperscript{a}All measurements taken after 15 days growth.

\textsuperscript{b}Each sugar was used at 20 g/l in the basal medium.
cellobiose, and raffinose reduce cell division and dry weight accumulation in suspension cultures. Since comparisons of cell aggregate development are subject to question if large differences in growth between treatments exist, sucrose was the only carbon source studied in time course experiments for comparison with the influence of glucose.

The number of cells per aggregate reaches a maximum of slightly over 400 in the sucrose medium and 300 in the glucose medium (Figure 18). This appears to be due, in part, to a greater rate of cell division in sucrose cultures during the early stages since aggregate number is the same in both cases up to day 8. However, the difference in aggregate size observed after 12 days is clearly the result of the much reduced dissociation of cell aggregates in the presence of sucrose. By day 18 the average number of cells per aggregate is three times as great in sucrose despite the fact that total cell number is considerably lower. This inhibition of cell separation during the later stages of the culture cycle is the most striking feature of the presence of sucrose in the culture medium.

When auxin is omitted from the medium the cells used in this study show essentially no growth, but growth is greatly accelerated by the addition of 0.1 mg/l naphthaleneacetic acid (NAA) (Table 6). As shown in Table 6 and Figure 19 cell separation is promoted by raising the NAA concentration to 1.0 mg/l. Increasing the NAA level to 5.0 mg/l does not affect
Figure 18. Cell number, aggregate number, and aggregate size as affected by the carbon source. solid circles = 2 per cent glucose open circles = 2 per cent sucrose
Cells per Aggregate

Aggregates per ml

Cells per ml ($\times 10^{-3}$)

DAYS

0 5 10 12 15 18

1000 300 500 1500 50 100
Figure 19. Cell number, aggregate number, and aggregate size as affected by NAA concentration.
open circles = 0.1 mg/l NAA
solid circles = 1.0 mg/l NAA
triangles = 5.0 mg/l NAA
the final aggregate size but does appear to slightly enhance separation during the phase of rapid cell division. The drastic reduction in aggregate number during the later stages of the culture cycle in 0.1 mg/l NAA indicates an inhibition of aggregate dissociation.

An inverse relationship between kinetin concentration and the degree of cell separation was observed at day 12 (Table 7); the largest cell aggregates occur in high kinetin media. Changes as a function of time in media containing 0, 0.5, and 10.0 mg/l kinetin show that kinetin delays cell separation (Figure 20). Cell separation in the absence of kinetin occurs most rapidly while cell division is at its highest rate (8 to 10 days). With 10.0 mg/l kinetin the greatest increase in aggregate number does not occur until after 12 days. Total cell number is not markedly affected by kinetin concentration.

The addition of various protein hydrolysates to the medium reduces average aggregate size in rose cell suspensions (Table 8). Since both acid and enzymatic digests of casein were found to have this effect, it is unlikely that the increased cell separation is due to tryptophan in the hydrolysate. Cell number is considerably higher in media containing protein hydrolysate. Casamino acids (Difco) was used in time course experiments at a concentration of 500 mg/l; in such experiments nitrate concentration was reduced by 50 per cent
Table 6. Growth and aggregate size as affected by NAA concentration

<table>
<thead>
<tr>
<th>NAA conc. (mg/1)</th>
<th>Dry wt. per culture (mg)</th>
<th>Cell number per ml (x10^-3)</th>
<th>Aggregate size (cells per aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>8</td>
<td>---</td>
</tr>
<tr>
<td>0.1</td>
<td>193</td>
<td>80</td>
<td>194</td>
</tr>
<tr>
<td>1.0^c</td>
<td>256</td>
<td>99</td>
<td>103</td>
</tr>
<tr>
<td>5.0</td>
<td>222</td>
<td>94</td>
<td>87</td>
</tr>
</tbody>
</table>

^a All measurements taken after 14 days growth.
^b Basal medium contained glucose as the carbon source.
^c Control (standard NAA concentration).

Table 7. Aggregate size as affected by kinetin concentration

<table>
<thead>
<tr>
<th>Kinetin conc. (mg/1)</th>
<th>Aggregate size (cells per aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94</td>
</tr>
<tr>
<td>0.1</td>
<td>116</td>
</tr>
<tr>
<td>0.5^c</td>
<td>146</td>
</tr>
<tr>
<td>5.0</td>
<td>218</td>
</tr>
<tr>
<td>10.0</td>
<td>308</td>
</tr>
</tbody>
</table>

^a After 12 days growth.
^b Basal medium contained glucose as the carbon source.
^c Control (standard kinetin concentration).
Figure 20. Cell number, aggregate number, and aggregate size as affected by kinetin concentration.
open circles = 0 kinetin
solid circles = 0.5 mg/l kinetin
triangles = 10.0 mg/l kinetin
Table 8. Cell number and aggregate size\textsuperscript{a} as affected by protein hydrolysates

<table>
<thead>
<tr>
<th>Protein hydrolysate\textsuperscript{b}</th>
<th>Cell number per ml (x10^{-3})</th>
<th>Aggregate size (cells/aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>98</td>
<td>130</td>
</tr>
<tr>
<td>Casamino acids\textsuperscript{c,e}</td>
<td>123</td>
<td>60</td>
</tr>
<tr>
<td>(casein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Edamin\textsuperscript{d,f}</td>
<td>146</td>
<td>81</td>
</tr>
<tr>
<td>(lactalbumin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Z Amine Type AS\textsuperscript{d,f}</td>
<td>152</td>
<td>64</td>
</tr>
<tr>
<td>(casein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hy-Case S.F.\textsuperscript{d,e}</td>
<td>115</td>
<td>43</td>
</tr>
<tr>
<td>(casein)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soy Peptone Powder\textsuperscript{d,f}</td>
<td>129</td>
<td>45</td>
</tr>
</tbody>
</table>

\textsuperscript{a}After 13 days growth.

\textsuperscript{b}All were added at 300 mg/l.

\textsuperscript{c}Difco Laboratories.

\textsuperscript{d}Sheffield Chem. Co.

\textsuperscript{e}Acid digest.

\textsuperscript{f}Enzymatic digest.
Table 9. Cell number and aggregate size as affected by casein hydrolysate and nitrate concentration

<table>
<thead>
<tr>
<th>Casein hydrolysate (mg/l)</th>
<th>Nitrate conc. (mM)</th>
<th>Cell number per ml ($\times 10^{-3}$)</th>
<th>Aggregate size (cells per aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.2$^c$</td>
<td>92</td>
<td>140</td>
</tr>
<tr>
<td>500</td>
<td>0$^d$</td>
<td>42</td>
<td>36</td>
</tr>
<tr>
<td>500</td>
<td>12.1$^d$</td>
<td>90</td>
<td>43</td>
</tr>
<tr>
<td>500</td>
<td>24.2</td>
<td>125</td>
<td>64</td>
</tr>
</tbody>
</table>

$^a$After 12 days growth.

$^b$Difco Laboratories.

$^c$Control nitrate concentration.

$^d$Chloride was substituted for nitrate in low nitrate media.

and replaced by chloride. This results in a more similar rate of cell division and further enhances cell separation (Table 9). The presence of casein hydrolysate greatly promotes the separation of proliferating cells; large aggregates do not occur at any stage in the culture cycle (Figure 21). Cell separation enhanced by casein hydrolysate occurs during the phase of most rapid division and is not accompanied by extensive cell enlargement (Figure 22). Cells grown in media containing casein hydrolysate are much more uniform in size and shape than those in the standard medium; this difference is
Figure 21. Cell number, aggregate number, and aggregate size as affected by casein hydrolysate.

- Solid circles = control
- Open circles = $1/2 \times$ control nitrate + 500 mg/l casein hydrolysate
Figure 22. Rose cells grown in medium containing 1/2 x control nitrate and 500 mg/l casein hydrolysate. (x85).
Table 10. Effect of adding a different sugar to glucose- and sucrose-grown cell suspensions prior to the period of aggregate dissociation

<table>
<thead>
<tr>
<th>Carbon source in medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Sugar added at 10 days&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Aggregate size at 12 days (cells per aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>sucrose</td>
<td>sucrose</td>
<td>225</td>
</tr>
<tr>
<td>sucrose</td>
<td>glucose</td>
<td>200</td>
</tr>
<tr>
<td>glucose</td>
<td>glucose</td>
<td>150</td>
</tr>
<tr>
<td>glucose</td>
<td>sucrose</td>
<td>210</td>
</tr>
</tbody>
</table>

<sup>a</sup>Used at 20 g/l.

<sup>b</sup>0.45 per cent; filter-sterilized.

especially noticeable after day 10. The addition of 200 mg/l glutamine to the standard medium partially replaces the effect of the protein hydrolysate, resulting in an average of 82 cells per aggregate at day 8, whereas the presence of protein hydrolysate results in 35 cells per aggregate.

Attempts were made to induce short term changes in the degree of cell aggregation by adding substances that enhance cell separation to growing cultures. The addition of 0.45 per cent glucose to sucrose-grown cells at day 10 does not overcome the sucrose inhibition of aggregate dissociation (Table 10). However, if 0.45 per cent sucrose is added to 10-day control cultures, normal cell separation appears to be
arrested. Further evidence of short term effects was achieved by the addition of 1.0 mg/l NAA to 7-day low (0.1 mg/l) NAA cultures. Such treatments promote a decrease in average aggregate size during the next 72 hr (Table 11). However, the added auxin also increases cell division activity. Therefore, it is not clear whether the decrease in average aggregate size is due to the separation of newly formed cells or to an auxin-induced aggregate dissociation. Clarification of this point requires the demonstration of an auxin effect on aggregate size in the absence of increased cell proliferation. Since cell number is nearly constant after day 12, 1.0 mg/l NAA was added to 13-day rose cell suspensions initially containing 0.1 mg/l NAA. This treatment reduces aggregate size, but, despite the age of the cultures, cell division is greatly stimulated (Table 12). Casein hydrolysate (500 mg/l) added to 7-day rose cell suspensions does not result in a decreased number of cells per aggregate (Table 13).

Tissue differences

In addition to alterations in the culture medium, the degree of cell separation is influenced by the nature of a particular tissue. During the course of this study, a variant strain was isolated from the normal callus. It appeared during the growth of a stock culture on agar-solidified medium; a portion of this stock callus appeared more translucent and more friable than the rest of the tissue. This isolate
Table 11. Aggregate size as affected by the addition of NAA\(^a\) to 7-day-old rose cell suspensions\(^b\)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Cell number per ml((x10^{-3}))</th>
<th>Aggregate size(\text{cells per aggregate})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>control</strong>(^b)</td>
<td><strong>+NAA</strong></td>
</tr>
<tr>
<td>0</td>
<td>33</td>
<td>--</td>
</tr>
<tr>
<td>24</td>
<td>43</td>
<td>50</td>
</tr>
<tr>
<td>48</td>
<td>72</td>
<td>83</td>
</tr>
<tr>
<td>72</td>
<td>96</td>
<td>91</td>
</tr>
</tbody>
</table>

\(^a\)1.0 mg/l filter-sterilized NAA.

\(^b\)Cells grown in medium containing 0.1 mg/l NAA.

Table 12. Aggregate size as affected by the addition of NAA\(^a\) to 13-day-old rose cell suspensions\(^b\)

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Cell number per ml((x10^{-3}))</th>
<th>Aggregate size(\text{cells per aggregate})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>control</strong>(^b)</td>
<td><strong>+NAA</strong></td>
</tr>
<tr>
<td>0</td>
<td>97</td>
<td>---</td>
</tr>
<tr>
<td>48</td>
<td>87</td>
<td>127</td>
</tr>
<tr>
<td>72</td>
<td>90</td>
<td>124</td>
</tr>
</tbody>
</table>

\(^a\)1.0 mg/l filter-sterilized NAA.

\(^b\)Cells grown in medium containing 0.1 mg/l NAA.
Table 13. Aggregate size as affected by the addition of casein hydrolysate to 7-day-old rose cell suspensions

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Cell number per ml (x10^-3)</th>
<th>Aggregate size (cells per aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>+C.H.</td>
</tr>
<tr>
<td>0</td>
<td>41</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>320</td>
<td>---</td>
</tr>
<tr>
<td>36</td>
<td>61</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>290</td>
<td>330</td>
</tr>
<tr>
<td>72</td>
<td>83</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>190</td>
</tr>
</tbody>
</table>

500 mg/l filter-sterilized Casamino acids (Difco).

has been labeled strain B and has been subcultured 20 times at 30-day intervals without changing appearance or identity; when cultured on solid medium it forms an extremely loose and watery callus. On solid medium it grows as rapidly as the normal strain, but total cell number achieved in suspension culture is much less than in the control (Figure 23).

The striking feature of strain B is its uniform appearance in liquid culture. Cells of this tissue show a high degree of separation throughout the culture cycle (Figure 23). The increase in aggregate number follows a time course that is very similar to the increase in cell number. As a result the average number of cells per aggregate remains very low; large aggregates of tightly packed cells do not occur in strain B suspension cultures. Strain B cells are rather large.
Figure 23. Cell number, aggregate number, and aggregate size in suspensions of the normal strain (solid circles) and the variant, strain B (open circles).
(Figure 24) and cell size remains fairly constant throughout the culture cycle.

**Changes in cell wall composition**

Analysis of the neutral and uronic acid components of the non-cellulosic polysaccharide fraction of the wall together with the estimate of cellulose content resulted in a total recovery of 87.4 per cent of the isolated rose cell walls (Table 14). The composition of an extracellular polysaccharide (precipitated from the culture medium with 80 per cent ethanol) differs greatly from that of the cell wall. The low content of galacturonic acid and the absence of cellulose in the extracellular material are particularly striking.

Since the degree of cell aggregation is influenced by culture age and modifications of the nutrient medium, the effects of these factors on wall composition and wall changes were determined. It was felt that the involvement of the cell wall in aggregate development might be reflected in quantitative changes in wall composition. Specific changes in wall composition occur during the 18-day culture cycle (Figure 25). Galactose decreases by one-third between days 12 and 15. Chronologically, this is correlated with the separation of cells which occurs after cell division has ceased. Arabinose undergoes a similar but less pronounced decrease during the same period. The specificity of the changes in galactose and, to a lesser degree, arabinose is particularly evident after
Figure 24. Suspension-cultured strain B cells. (x85).
Table 14. Composition\textsuperscript{a} of walls isolated from cultured\textsubscript{b} rose cells and of an extracellular polysaccharide\textsuperscript{c}

<table>
<thead>
<tr>
<th>Component</th>
<th>Rose cell walls</th>
<th>Extracellular Polysaccharide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-cellulosic fraction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rhamnose</td>
<td>2.9</td>
<td>2.5</td>
</tr>
<tr>
<td>fucose</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>arabinose</td>
<td>6.0</td>
<td>13.2</td>
</tr>
<tr>
<td>xylose</td>
<td>6.0</td>
<td>17.8</td>
</tr>
<tr>
<td>mannose</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>galactose</td>
<td>12.6</td>
<td>25.3</td>
</tr>
<tr>
<td>glucose</td>
<td>3.1</td>
<td>18.7</td>
</tr>
<tr>
<td>glucuronic acid</td>
<td>0.9</td>
<td>3.3</td>
</tr>
<tr>
<td>galacturonic acid</td>
<td>20.4</td>
<td>4.7</td>
</tr>
<tr>
<td>Cellulose\textsuperscript{c}</td>
<td>33.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Total</td>
<td>87.4</td>
<td>87.9</td>
</tr>
</tbody>
</table>

\textsuperscript{a}All values represent mg sugar per 100 mg starting material.

\textsuperscript{b}Obtained from culture medium by precipitation with 80 per cent ethanol.

\textsuperscript{c}Anthrone determination on residue remaining after TFA:enzyme hydrolysis.
Figure 25. Composition of the non-cellulosic polysaccharide fraction of rose cell walls during an 18-day culture cycle in the standard medium.
Figure 25. (continued).
mg sugar per 100 mg wall

rhamnose

xylose

galacturonic acid

galacturonic acid

AGE (DAYS)

0 5 10 15 18
12 days. All other components of the non-cellulosic fraction of the wall remain relatively unchanged, although glucose increases slightly after transfer to fresh medium and xylose increases slightly around day 10.

After TFA:enzyme hydrolysis, an insoluble cell wall residue remains. The contribution of this residue to total wall weight during the culture cycle is shown in Table 15.

Changes in the composition of walls isolated from cells grown with sucrose as a carbon source (Figure 26) are similar to those of the control (Figure 25). However, wall galactose is slightly higher in sucrose-grown cells throughout the culture cycle; final (day 18) levels of galactose and arabinose are higher in walls isolated from the sucrose cells.

When grown in the low NAA medium, the cells show a less pronounced decrease in wall galactose (Figure 27). The effect of low auxin on the galactose decrease is noticeable only because changes in the amounts of other sugars are unaffected by this treatment. Cells grown in 10.0 mg/l kinetin maintain somewhat higher levels of wall galactose between 12 and 15 days (Figure 28); however, galactose content does decrease sharply after day 15. Another effect of high kinetin is to substantially increase glucose in the non-cellulosic polysaccharide fraction of the wall.

Growth of cells in a medium lacking kinetin results in an increased level of galactose in the cell wall (Figure 29);
Table 15. "Cellulose"\(^a\) content of rose cell walls during 18-day culture cycle in the standard medium

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>0</th>
<th>5</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>15</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellulose</td>
<td>29.6</td>
<td>30.8</td>
<td>30.8</td>
<td>32.9</td>
<td>37.3</td>
<td>39.6</td>
<td>33.8</td>
</tr>
</tbody>
</table>

(mg/100 mg wall)

\(^a\) Anthrone determination on residue remaining after TFA:enzyme hydrolysis.
Figure 26. Composition of the non-cellulosic polysaccharide fraction of rose cell walls during an 18-day culture cycle in sucrose medium.
The diagram shows the concentration of various sugars (galactose, arabinose, glucose, fucose) in mg per 100 mg wall over different ages (days). The y-axis represents the concentration in mg sugar per 100 mg wall, while the x-axis represents age in days. The graph indicates the changes in sugar concentration over time, with specific peaks and troughs for each sugar type.
Figure 26. (continued).
mg sugar per 100 mg wall

galacturonic acid

galacturonic acid

rhamnose

xylose

AGE (DAYS)

0 5 8 10 12 15 18
Figure 27. Composition of the non-cellulosic polysaccharide fraction of rose cell walls during an 18-day culture cycle in low (0.1 mg/l) NAA medium.
Figure 27. (continued).
Figure 28. Composition of the non-cellulosic polysaccharide fraction of rose cell walls during an 18-day culture cycle in high (10.0 mg/l) kinetin medium.
Figure 28. (continued).
Figure 29. Composition of the non-cellulosic polysaccharide fraction of rose cell walls during an 18-day culture cycle in medium lacking kinetin.
Figure 29. (continued).
this is established early and maintained throughout the period of most rapid growth. Galactose does decrease after day 12 but remains higher than the control at day 18. Walls from cells cultured in minus kinetin medium also show an elevated arabinose content.

The presence of casein hydrolysate also produces cells with walls that are high in galactose (Figure 30). Walls from 10 to 15 day-old casein hydrolysate cells contain 18 to 20 per cent galactose; control cell walls contain about 11 per cent galactose at day 15. The presence of casein hydrolysate, besides increasing the amount of galactose in the wall, also causes wall arabinose to increase rather than decrease towards the end of the culture cycle.

The composition of the non-cellulosic fraction of strain B cell walls (Figure 31) differs from that of the normal strain in several ways. The walls of these cells contain an increased level of galactose, and, unlike the normal strain, galactose content does not decrease after day 12. In addition to the galactose difference, galacturonic acid, arabinose, and xylose are present in lower amounts in strain B cell walls than those of the normal strain.

There is one consistent feature of cells which undergo separation readily during the period of most rapid division. That is, the factors that promote the separation of these cells also lead to increased levels of wall galactose. This
Figure 30. Composition of the non-cellulosic polysaccharide fraction of rose cell walls during an 18-day culture cycle in casein hydrolysate medium.
Figure 30. (continued).
Figure 31. Composition of the non-cellulosic polysaccharide fraction of strain B rose cell walls during an 18-day culture cycle in standard medium.
Figure 31. (continued).
mg sugar per 100 mg wall

rhamnose

xylose

galacturonic acid

AGE (DAYS)

galacturonic acid
is illustrated in Figure 32 which compares the galactose content of walls isolated from normal cells cultured in minus kinetin and plus casein hydrolysate media and from cells of the variant tissue, strain B to that of control cell walls.

**Extracellular glycosidase activity**

Cell wall-modifying enzyme activity is an aspect of cell surface biochemistry that may be important in aggregate development. It was felt that cell surface enzymes might be responsible for the changes in wall composition described above. Furthermore, enzymes may mediate changes at the cell surface that cannot be detected by quantitative component analyses of wall hydrolysates. The extract obtained by suspending unbroken cells in 0.5 M sodium citrate presumably removes enzymes that are in some way associated with the cell wall. The enzyme preparation obtained in this way catalyzes the release of reducing groups from isolated rose cell walls (Figure 33). Cell walls incubated for 20 hr in the presence of surface extract contain slightly less galacturonic acid, 7 per cent less arabinose and 13 per cent less galactose than walls treated with the boiled enzyme control (Table 16).

The activity of extracellular enzyme preparations against various glycosides and polysaccharides is shown in Table 17. The cell surface extract shows β-glucosidase, β-galactosidase, and laminarinase activities. The culture medium is active primarily against the β-galactoside model substrate. Enzyme-
Figure 32. Galactose content of walls isolated from cells that undergo extensive separation prior to the cessation of cell division. The composition of control cell walls is indicated by the solid circles.
Figure 33. Release of reducing groups from isolated rose cell walls incubated in a 0.5 M sodium citrate extract of intact cells.
Table 16. Effect of cell surface extract on the composition of isolated rose cell walls after a 20 hr incubation period

<table>
<thead>
<tr>
<th>Sugar (mg/100 mg wall)</th>
<th>galacturonic acid</th>
<th>fucose</th>
<th>rhamnose</th>
<th>glucose</th>
<th>xylose</th>
<th>arabinose</th>
<th>galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boiled enzyme</td>
<td>23.0</td>
<td>1.4</td>
<td>3.4</td>
<td>4.1</td>
<td>6.0</td>
<td>5.6</td>
<td>13.2</td>
</tr>
<tr>
<td>Enzyme treated</td>
<td>22.1</td>
<td>1.5</td>
<td>3.4</td>
<td>4.2</td>
<td>6.1</td>
<td>5.2</td>
<td>11.5</td>
</tr>
</tbody>
</table>
Table 17. Specific activity of the cell surface extract and the culture medium against various substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (umoles/hr/mg prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Surface extract</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-glucoside</td>
<td>19.7</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-galactoside</td>
<td>8.2</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-xyloside</td>
<td>0.8</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-glucoside</td>
<td>0.6</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-galactoside</td>
<td>1.5</td>
</tr>
<tr>
<td>p-nitrophenyl-α-D-xyloside</td>
<td>0.2</td>
</tr>
<tr>
<td>arabinan\textsuperscript{a}</td>
<td>1.9</td>
</tr>
<tr>
<td>carboxymethylcellulose\textsuperscript{b}</td>
<td>0.6</td>
</tr>
<tr>
<td>laminarin\textsuperscript{c}</td>
<td>11.3</td>
</tr>
<tr>
<td>lichenan\textsuperscript{a}</td>
<td>2.2</td>
</tr>
<tr>
<td>nigeran\textsuperscript{a}</td>
<td>0.0</td>
</tr>
<tr>
<td>xylan\textsuperscript{a}</td>
<td>1.7</td>
</tr>
<tr>
<td>Na polypectate\textsuperscript{d}</td>
<td>1.0</td>
</tr>
<tr>
<td>pectin\textsuperscript{e}</td>
<td>0.5</td>
</tr>
<tr>
<td>polygalacturonic acid\textsuperscript{e}</td>
<td>1.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Koch-Light Laboratories.

\textsuperscript{b}Hercules, Inc.

\textsuperscript{c}Pierce Chem. Co.

\textsuperscript{d}Nutritional Biochem. Corp.

\textsuperscript{e}Sunkist Co.
mediated release of reducing groups from three galacturonic acid substrates was found to be negligible; the same results were obtained when these substrates were used in viscometric assays.

The presence of glycosidases at the cell surface was verified by incubating water-washed intact rose cells in buffered substrate solutions. The β-galactoside and β-glucoside model substrates were rapidly hydrolyzed by suspensions of whole cells. Since reaction times were short (10 minutes), substrate uptake and subsequent hydrolysis by intracellular enzymes seems an unlikely explanation for this activity.

Whole cell suspensions were assayed before and after extraction; it was found that 0.5 M citrate removes about 30 per cent of the cell surface glycosidase activity.

The ratio of β-glucosidase to β-galactosidase activity was the same in the surface preparations from cells grown under a variety of cultural conditions. This suggested that the two activities may reside in one protein; this possibility was tested in several ways. The first of these involved a comparison of the heat stability of β-glucosidase and β-galactosidase. Samples of the cell surface extract were placed for various times in a water bath maintained at 55 C. The consequent decrease in activity as a function of time against both substrates follows a similar pattern (Figure 34). It was noted that sodium citrate (pH 4.6) was an effective inhibitor of the glycosidase obtained from the cell surface.
Figure 34. Heat stability of the β-glucosidase and β-galactosidase activities of the rose cell surface extract.
Table 18. Effect of 2 mM nojirmycin on the enzyme activity of the cell surface extract.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Per cent of control activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-nitrophenyl-β-D-glucoside</td>
<td>43</td>
</tr>
<tr>
<td>p-nitrophenyl-β-D-galactoside</td>
<td>48</td>
</tr>
<tr>
<td>laminarin</td>
<td>57</td>
</tr>
<tr>
<td>isolated rose cell walls</td>
<td>68</td>
</tr>
</tbody>
</table>

Increasing concentrations of sodium citrate inhibit β-glucosidase and β-galactosidase to an identical degree (Figure 35). Activity against the two substrates is also similarly inhibited by nojirmycin (5-amino-5-deoxy-D-glucopyranose) (Figure 36). Table 18 compares the effects of 2 mM nojirmycin on the activity of the cell surface extract against β-galactoside, β-glucoside, laminarin, and isolated rose cell walls.

It was difficult to accurately assess the effect of nojirmycin on laminarinase and cell wall-degrading activity. Nojirmycin is a reducing sugar and therefore reacts with the dinitrosalicylate reagent. Also, to detect activity against these substrates, a highly concentrated enzyme preparation must be used; it may be that this masks the nojirmycin inhibition.

While both β-glycosidase activities extracted from the cell wall appear to be a property of the same protein, this enzyme appears to be quite different than the β-galactosidase present in the culture medium. The medium enzyme is only
Figure 35. Sodium citrate inhibition of the β-glucosidase and β-galactosidase activities of the rose cell surface extract.
Figure 36. Nojirmycin inhibition of the β-glucosidase and β-galactosidase activities of the rose cell surface extract.
slightly inhibited by high levels of sodium citrate (Figure 37) and is unaffected by nojirimycin (Figure 38). In addition, the β-galactosidase in the culture medium follows an entirely different time course than that of the cell surface (Figure 41).

Since nojirimycin so effectively inhibits cell surface glycosidase activity it was of interest to determine its in vivo effect on cell aggregate dissociation. This was done by exposing 12-day suspension cultures to 1 mM nojirimycin. Cultures of this age are characterized by increasing aggregate number in the absence of cell division activity (Figure 9). Nojirimycin inhibits the increase in aggregate number that occurs after day 12 (Figure 39). By inhibiting enzymes capable of modifying isolated cell wall material, nojirimycin may indirectly influence the wall changes normally occurring in rose cell suspensions following day 12. Evidence supporting this hypothesis is shown in Figure 40; nojirimycin clearly affects wall composition. Specifically, rose cells show a much less pronounced decrease in wall galactose after nojirimycin treatment; the amounts of other wall components are not affected.

Because wall composition (Figure 25) and the degree of cell aggregation (Figure 9) change with culture age, the influence of age on enzyme activity was determined. During the 18-day culture cycle, it was found that changes in the specific activity of the cell surface glycosidases (Figure 41) are correlated with the loss of wall galactose and with cell
Figure 37. Sodium citrate inhibition of the cell surface and culture medium $\beta$-galactosidase activities.
Figure 38. Nojirmycin inhibition of the cell surface and culture medium β-galactosidase activities.
Figure 39. Inhibition of aggregate dissociation by the addition of nojirimycin to 12-day-old rose cell suspensions.
solid circles = control
open circles = 1 mM nojirimycin added at day 12
Figure 40. Effect of nojirmycin on changes in rose cell wall composition.
- solid circles = control
- open circles = 1 mM nojirmycin added at day 12
mg sugar per 100 mg wet weight

AGE (DAYS)

galactose

arabinose

glucose

fucose
Figure 40. (continued).
Figure 41. Specific activity (μM product formed per hr per mg protein) of extracellular β-glycosidase activities during an 18-day culture cycle in the standard medium. Substrates were p-nitrophenyl-β-glycosides.
aggregate dissociation. The β-galactosidase and β-glucosidase both decrease slightly following transfer to fresh nutrient medium. Specific activity remains low throughout the period of aggregate formation and rapid division. However, during the stationary phase of growth, cell surface glycosidase activity shows a 10-fold increase. Most of this increase occurs between days 12 and 15. In contrast, the specific activity of the culture medium β-galactosidase increases until day 12 and then falls off rapidly.

The fact that the changes in wall galactose and surface β-galactosidase begin as a culture enters the stationary phase suggests that these changes may be a cellular response to the depletion of some nutrient. Depletion of the carbon source might be expected at this stage in the culture cycle. Since the standard carbon source was glucose, its concentration in the medium was easily followed by using the Glucostat reagent (Worthington Biochem. Corp.). The concentration of glucose in the medium decreases most sharply between days 10 and 12 (Figure 42). This is immediately prior to the pronounced increase in β-galactosidase activity that occurs between days 12 and 15. Also, it is after day 12 that wall galactose decreases by 30 to 35 per cent. By day 15, no glucose could be detected in the culture medium.

To further examine the possible relationship between glucose level and cell surface glycosidase activity, aliquots of 11-day-old cultures were separated from the culture medium
Figure 42. Changes in glucose concentration in the medium, cell surface β-galactosidase activity, and wall galactose content during an 18-day culture cycle in the standard medium.
glucose in medium (‰) —

AGE (DAYS)

wall galactose —

β-galactosidase S.A. —

0 10 20

0 5 10 15 20

0 5 10 15 18
Table 19. Cell surface β-glycosidase activity as affected by the transfer of cells to media containing 0 or 2 per cent glucose

<table>
<thead>
<tr>
<th>Time after transfer (hr)</th>
<th>Surface enzyme activity (µmoles/hr/mg prot.)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β-glucosidase</td>
<td>β-galactosidase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+glucose</td>
<td>-glucose</td>
<td>+glucose</td>
<td>-glucose</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>8.0</td>
<td>8.0</td>
<td>4.0</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>13.6</td>
<td>17.7</td>
<td>2.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>15.9</td>
<td>24.0</td>
<td>3.6</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>72</td>
<td>17.8</td>
<td>28.2</td>
<td>5.6</td>
<td>13.2</td>
<td></td>
</tr>
</tbody>
</table>

*aAliquots of 11-day-old cell suspensions.

by centrifugation and resuspended in 15 ml of fresh nutrient media which contained either 0 or 2 per cent glucose. These suspensions were then incubated on a shaker at 27 C. Surface extracts were subsequently obtained from the treated cells after 18, 45, and 72 hr. Enzyme activity is slightly higher in the minus glucose suspensions after 18 hr; this difference is clearly established after 72 hr (Table 19). The presence of glucose clearly suppresses glycosidase activity.

To further explore the relationship between cell surface β-galactosidase and changes in wall galactose (Figure 42), the effect of medium composition on enzyme activity was determined. For this purpose, medium modifications were selected that influence wall galactose content in different ways. These
included the use of sucrose as a carbon source, which has little effect on changes in galactose (Figure 26); low NAA, in which the galactose decrease is delayed and less pronounced (Figure 27); and the addition of casein hydrolysate, which results in a substantially higher level of wall galactose (Figure 30). The time course of β-galactosidase activity at the surface of cells grown in glucose and sucrose is nearly identical (Figure 43). However, in the culture medium containing 0.1 mg/l NAA the enzyme activity is significantly affected (Figure 44). Most of the increase in specific activity does not occur until after 15 days. Furthermore, the activity of the surface glycosidase does not increase nearly as much as in control cultures (1.0 mg/l NAA). The presence of casein hydrolysate (500 mg/l) in the culture medium also alters the pattern of enzyme appearance; in this treatment enzyme activity is greatly reduced (Figure 45). At day 12, the β-galactosidase activity of control and treated cells is 68 and 10 μmoles product per hr per mg protein respectively; by day 18 the activity of casein hydrolysate cells is only 28. The activity of the surface extract against the β-glucoside substrate, although not shown in Figures 43, 44, and 45, was found to exhibit the same pattern of change in each medium as does β-galactosidase activity.

To test the hypothesis that enzymatic activity associated with the cell wall might be involved in aggregate dissociation in culture, the cell surface extract was tested for its
Figure 43. Specific activity (µM product formed per hr per mg protein) of surface β-galactosidase of rose cells in glucose (solid diamonds) and sucrose (open circles) media.
Figure 44. Specific activity (μM product formed per hr per mg protein) of surface β-galactosidase of rose cells as affected by NAA concentration.
Figure 45. Specific activity (μM product formed per hr per mg protein) of surface β-galactosidase of rose cells as affected by casein hydrolysate.
ability to bring about cell separation. In these experiments, 10 to 12 day-old suspension cultures were diluted with an equal volume of distilled water; the medium was removed from 3 ml aliquots of the diluted cell suspensions by centrifugation. The cells were then resuspended in an enzyme solution prepared by dissolving the lyophilized surface extract in conditioned culture medium at approximately 100 µg protein per ml. Boiled enzyme solutions were used as controls. Treated suspensions were incubated with shaking at 27 C for 12 hr. Total aggregate number was determined and used to evaluate cell separation. In three separate experiments aggregate number was 8 to 13 percent higher in the enzyme-treated suspensions than in the boiled enzyme controls. Commercial preparations of various hydrolytic enzymes were tested under similar conditions (Table 20). Sigma pectinase, as expected, results in nearly complete cell separation. Cellulase (Worthington) causes extensive cell degradation and it was not possible to assess its cell-separating activity. Purified preparations of β-glucosidase (almonds; Worthington) and β-galactosidase (E. coli; Worthington) have no effect on aggregate number in the system described.

Attempts to induce spontaneous cell adhesion

Efforts were made to cause separated cells of Paul's Scarlet rose tissue to adhere upon contact. The purpose of these experiments was to assess the feasibility of applying
Table 20. Cell-separating activity of the cell surface extract and various commercial enzyme preparations.

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Per cent increase in aggregate number per hr per mg prot.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pectinase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2000</td>
</tr>
<tr>
<td>Cellulase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>--</td>
</tr>
<tr>
<td>β-galactosidase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>β-glucosidase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>Rose cell surface extract</td>
<td>11</td>
</tr>
</tbody>
</table>

<sup>a</sup>Sigma Chem. Co.

<sup>b</sup>Worthington Biochem. Corp.

Some of the techniques used with animal cells to the study of cultured plant cells. For this purpose it was necessary to first obtain large numbers of cells in a relatively dissociated state. This was accomplished by passing cell suspensions through a sieve having a pore size of 0.105 mm. Retained cell aggregates were discarded and the resulting suspension was concentrated by low speed centrifugation. It was composed of single cells, cell pairs, and clusters of less than ten cells. Various attempts were then made to induce spontaneous adhesion and consequent aggregate formation from the suspensions of separated cells. In each of the experiments described here, the treated cell suspensions were incubated
on a rotary shaker operated at 100 RPM to ensure that the cells did contact each other.

One of the components that may be important in the intercellular bonds of intact plant tissue is calcium. Calcium may participate in intercellular bonds by cross-linking uronic acid residues in the plant cell wall; the number of calcium bridges is limited by the degree to which the uronic acid sites are methyl esterified. It was felt that separated cells might be made to adhere to one another by increasing the number of free carboxyl groups at the cell surface and providing calcium ions. To test this idea aliquots of separated cells were exposed to pectin methyl esterase (PME) (Sigma) at concentrations of 0, 0.01, 0.02, 0.1, and 0.2 mg per ml and calcium at 0, 3, and 6 μM; all combinations were tested. The pH was adjusted to 6.0 and the treated suspensions were incubated at 30 C for up to 48 hr. The results of several experiments with PME and calcium were, however, entirely negative; no accumulation of cells into larger aggregates was observed. The effect of calcium concentration in the medium was also tested in growth experiments. It was found that increased levels of calcium promote dry weight accumulation by day 12, but do not affect the degree of cell aggregation. The effect of pH was also tested in culture experiments; pH of the medium was adjusted to 4.0, 5.5, or 7.0 prior to inoculation. Growth (dry weight) is not affected at pH 4.0, but is greatly reduced at a pH of 7.0; cell aggregation is apparently
not influenced by the pH of the culture medium.

In other experiments, various polysaccharides were added to suspensions of separated cells in attempts to induce aggregate formation. The rationale was that certain polysaccharides might serve as an intercellular matrix in the formation of cell aggregates. Pectin N. F. (Sunkist), polygalacturonic acid (Sunkist), carboxymethylcellulose (Hercules), gum arabic (Baker), or galactomannan (Sigma) was added at 1.0 per cent to 10 ml suspensions of the separated rose cells; treated suspensions were observed after 12 and 24 hr. None of the polysaccharides tested resulted in cell aggregate formation.

Since lectins, which aggregate animal cells, are known to bind carbohydrates, it seems reasonable to suggest that their presence could also cause separated plant cells to adhere to each other. The readily available and most commonly used lectin, concanavalin A, was added to separated cell suspensions at a concentration of 1 mg/ml in 0.02 M phosphate buffer (pH 6.9) and 0.01 M sodium chloride. However, concanavalin A did not promote cell adhesion during the 12 hr incubation period.
DISCUSSION

The results reported here demonstrate that the degree of cell aggregation is a feature of suspension culture development that is under rather precise control. The salient fact is that formation and dissociation of cell aggregates are controlled temporally and can be regulated by altering the nutrient medium. The ability to control aggregate size makes possible a correlation between the degree of cell aggregation and biochemical changes at the cell surface.

The method used in this investigation for describing aggregate size has permitted a more quantitative description of aggregate development than can be made on the basis of sieving techniques (Sussex and Clutter, 1967) and qualitative observations (Liau and Boll, 1971). It is essential that aggregate size be described in precise terms exclusive of the confounding effect of cell size variation if the basic mechanisms of the cellular interactions that control aggregate formation are to be studied and understood.

The rose tissue used in this study, when grown in the standard medium, follows a developmental pattern that is typical of most plant suspension cultures. That is, cell aggregates tend to increase in size during the period of greatest cell division activity and dissociate as the rate of division slows and during the stationary phase of growth. This is consistent with the findings of Torrey, et al. (1962);
Henshaw, et al. (1966); Mehta, et al. (1967); and Liau and Boll (1971). But apparently not all cultured cells follow this pattern as Sussex and Clutter (1967) reported that aggregate dissociation is not restricted to any particular phase of the growth cycle in suspension cultures of *Eucalyptus camaldulensis*. This contrast exhibited by *Eucalyptus* merits further study.

While rose cells exhibit a rather typical developmental pattern of aggregate formation, this standard pattern may be drastically altered by rather simple means. Figure 46 summarizes the alternative pathways of aggregate development that may occur in cultures of Paul's Scarlet rose as a result of various experimental manipulations utilized in this study. In the standard medium, cells of the normal strain follow the pattern indicated by arrows 1 and 4. Aggregates form as a result of cell proliferation and dissociate as the rate of division slows. Arrows 1 and 3 depict aggregate development in media containing sucrose or low (0.1 mg/l) NAA; aggregate dissociation is largely inhibited. The lower portion of the model (arrows 2 and 5) represents aggregate development in three situations: normal cells in the absence of kinetin, normal cells in the presence of casein hydrolysate, and strain B cells in the standard medium. In each of these cases, cell separation accompanies rather than follows the period of rapid division; aggregate formation is thereby greatly reduced. It was observed, however, that some cell aggregates are formed in kinetin-free media early in the culture cycle.
Figure 46. Schematic illustration of various patterns of aggregate development. Each solid circle and oval represents approximately 30 cells; the shaded areas represent cell aggregates. This model is designed to indicate only the approximate number of cells per aggregate.
In view of the effect of sucrose and other sugars on the degree of cell aggregation (Table 5, Figure 18), it is of interest to point out that Nevins, et al. (1967) found that cell wall composition of suspension-cultured Acer cells is influenced by the kind of carbohydrate supplied as a carbon source. Furthermore, Cox and Gesner (1965) demonstrated that specific sugars in the medium interfere with cell aggregation in certain mammalian tissue cultures, perhaps, they suggest, by binding complementary sites on the cell surface. This led Nevins, et al. (1967) to consider that sugars in the medium might affect the plant cell's participation in surface interactions by inducing change in the polysaccharides of the cell surface although they provided no evidence to support this contention. The results reported here demonstrate that the carbon source does indeed modify aggregate formation. Wall composition is also affected, although only slightly, by the carbon source supplied to the cells (Figures 25 and 26). In addition, it is possible that sucrose induces wall changes which cannot be detected by quantitative analysis of wall polysaccharide components and which may act to reduce aggregate dissociation.

The fact that a low level of NAA suppresses cell separation is consistent with the findings of Torrey and Reinert (1961); Lamport (1964); Digby and Wareing (1966); and Hart, Woodcock, and Wilson (1970). The effect of auxin on cell elongation is known to involve cell wall changes (Loescher,
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1971); these changes are perhaps also involved in cell separation.

While sucrose and low NAA modify the behavior of cell aggregates at late stages of the growth cycle, other treatments cause a very different pattern of aggregate development. The contrasting modes of development (shown in Figure 46) appear to involve two types of cell separation. The first is the separation of cells which are in the phase of rapid division; the second is that which leads to the fragmentation of existing cell aggregates.

In the normal culture cycle, the large increase in aggregate number between days 8 and 12 may be the result of both types of cell separation. It is clear, however, that the increase in aggregate number that occurs after day 12 is due to aggregate dissociation since cell division activity has ceased. Aggregate dissociation is largely restricted to the stationary phase of growth. The fragmentation of rose cell aggregates is analogous to the cell separation that Street and Henshaw (1966) felt was precluded by a high rate of division.

Under certain conditions intercellular relationships are greatly altered and rose cells will separate readily despite a high rate of division. This type of separation is observed when the cells are cultured in the presence of casein hydrolysate. Also, in the kinetin-free medium, the early increase in aggregate number is most likely due to the separation of newly
formed cells. Possibly the presence of kinetin inhibits the separation of proliferating cells but not the dissociation of aggregates, since after 12 days in culture aggregate number increases at a similar rate independent of the initial kinetin concentration.

In addition to the difference in their relationship to mitotic activity, the two types of cell separation are quite dissimilar with respect to the involvement of cell expansion. Aggregate dissociation is usually accompanied by extensive cell enlargement (Digby and Wareing, 1966; Lamport, 1964; Henshaw, et al., 1966). A comparison of cell size and the number of cells per aggregate (Figure 10) during a normal rose culture cycle suggests that cell enlargement and this separation are related. This correlation has also been observed in sycamore cell cultures by Digby and Wareing (1966). Lamport (1964) implies that wall extension throughout the cell promotes separation and Henshaw, et al. (1966) also suggest that aggregate dissociation is dependent upon cell expansion. Unlike aggregate dissociation, the separation of rapidly dividing cells occurs without large increases in cell size.

Further evidence which supports the contention that cells which separate shortly after dividing do so in a different manner than those that separate after the formation of relatively large aggregates was obtained in cell wall studies. A basic difference in the mechanisms of the two types of cell separation is indicated by rather specific wall modifications
that accompany each. The changes involve primarily a difference in wall galactose content.

The establishment of a suspension of highly separated cells does not involve a drastic tissue reorganization when it occurs as shown in arrows 2 and 5 of Figure 46. This is particularly true of casein hydrolysate and strain B cells. A failure to organize (adhere after division), rather than the dissociation of aggregates, is involved in these cases. The data presented in Figure 32 suggest that an increase in wall galactose in the cell surface is, in part, related to the reduced tendency for proliferating cells to adhere to one another. A similar change may occur in control cultures between days 10 and 12; at this time dividing cells do appear to separate to some degree and there is a transient rise in wall galactose content (Figure 25).

In contrast to the separation of rapidly dividing cells, aggregate dissociation does involve a striking change in tissue organization. The transition from a suspension of mostly compact cell aggregates to one consisting of cells in loosely adhering groups, filamentous chains, pairs, etc., is relatively rapid. One would therefore expect relatively short term changes to occur at the cell surface. One aspect of the surface change involved in aggregate dissociation is an apparent loss of galactose from the wall. Cell separation leading to aggregate dissociation and the decrease in wall galactose are both confined to a particular stage in the
growth cycle. That arabinose content declines slightly during the same period suggests that the polysaccharide affected may be an arabinogalactan. Red Kidney bean root cells in culture are known to secrete an extracellular arabinogalactan (Hawes and Adams, 1972) and arabinogalactans are often associated with hemicellulosic polysaccharides of cell walls (Northcote, 1972). Additional evidence that a decrease in wall galactose is associated with aggregate dissociation was obtained by analyzing walls isolated from cells cultured in various modifications of the standard medium. For example, low NAA inhibits aggregate dissociation and also appears to suppress the wall modification which is expressed as a loss of galactose. Also, a high level of kinetin in the medium delays both of these changes. Furthermore, cultures in which aggregate dissociation does not occur because large aggregates are not formed in early stages of the culture cycle, do not show a marked decrease in wall galactose at later stages.

The changes in wall galactose that occur in sucrose-grown cells are not consistent with the idea that the decrease in wall galactose is causally related to aggregate dissociation. In the presence of sucrose, aggregates fail to dissociate, but wall galactose decreases, a pattern of wall change clearly correlated with dissociation in other media. However, it seems highly unlikely that such a change in tissue morphology requires or occurs concomitantly with a single physiological change. It may be that the loss of galactose is closely
related to aggregate dissociation, but that some additional condition for separation is not satisfied in the presence of sucrose.

The observation that two different types of cell-separating mechanisms exist in suspension-cultured plant cells is critical to the interpretation of the results of cell wall analyses which might otherwise seem contradictory. For, although in general both types of surface modifications are correlated with the same wall component, there is no reason to expect the precise nature of surface changes that prevent aggregate formation to be the same as those that are involved in aggregate dissociation.

The increase in wall galactose in rapidly dividing cells undergoing separation is probably directed by an entirely different set of physiological processes than is the decrease in galactose which accompanies aggregate dissociation. It may be that the increase in galactose (Figure 32) is under the influence of enzymes involved in wall synthesis. On the other hand, the nature of the wall change during aggregate dissociation suggests that it may be mediated by polysaccharide-degrading enzymes. The results presented here demonstrate that the surfaces of cultured rose cells contain enzymes with this latter capability.

Like aggregate dissociation and the loss of wall galactose, the increase in β-galactosidase is temporally controlled. The timing of the change in β-galactosidase activity may be
regulated by the glucose concentration in the medium. This type of response to nutrient depletion is fairly common, especially in the fungi. For example, Schizophyllum commune shows an increase in polysaccharide-degrading enzyme activity when the carbon source has been consumed and this increased enzyme activity appears to be of morphogenetic significance (Wessels, 1966). Also, Pyrenochnaeta terrestris shows a sequential production of pectinase and cellulase when deprived of a simple monosaccharide substrate (Horton and Keen, 1966). Phosphatase activity is induced in cultured cells of tobacco as phosphate is removed from the medium (Ueki and Sato, 1971).

The increased β-galactosidase activity in stationary phase cultures could account for the loss of galactose from the walls of non-dividing cells. The presence of sucrose, casein hydrolydate, or a low level of NAA affects wall galactose and β-galactosidase activity in a way which suggests that the two are directly related. This relationship is also indicated by the fact that a rather potent inhibitor of β-galactosidase (nojirimycin) prevents most of the galactose decrease. Thus, it is probable that the decrease in wall galactose is mediated by the cell surface β-galactosidase. The inhibition of aggregate dissociation by nojirimycin is perhaps also related to its effect on the β-galactosidase activity.

However, the relationship of this activity to the process of aggregate dissociation is a simple correlation and has not been demonstrated experimentally. A highly concentrated
solution containing the cell surface β-galactosidase does not effectively dissociate isolated cell aggregates. However, the exogenously supplied enzyme may have lost cell-separating activity during extraction and concentration or simply failed to reach its site of action.

Also somewhat perplexing are the results involving the uronic acid portion of the cell wall. The cells of strain B, which apparently are far less adhesive than those of the normal strain, contain substantially less galacturonic acid in the wall. Fuller and Grant (1968) reported a similar correlation between the content of pectic substances and the friability of two clones of bean callus tissue. This observation is consistent with the idea that polyuronides stabilize plant tissue by crosslinking via calcium salt bridges. However, enzymes that degrade galacturonic acid polymers are apparently absent from the rose cell surface. This is of particular interest because of the profound cell-separating activity of commercial pectinase preparations. It is, of course, possible that pectic enzymes are present which are not active against the substrates tested, but pectinase has not been found in any tissue cultures which have been examined for this activity (Mandels, et al., 1967; Krikorian and Steward, 1969). These authors assayed extracts of carrot, tobacco, peanut, Haplopappus, bean, lettuce, and pepper tissue cultures. Furthermore, in the rose studies reported here, the level of galacturonic acid content in the wall remains unchanged throughout the culture cycle of
both strains of tissue and is not markedly affected by medium alterations that influence cell aggregation.

Admittedly the analyses of wall composition and enzyme activity reported here allow only a superficial examination of the cell surface. It must be considered, for instance, that the pectic substances may undergo significant structural modification without showing a net change in the number of uronic acid residues. Thus, this investigation of the involvement of the wall in plant cell surface interactions is obviously of a preliminary nature. Nevertheless, studies of physiological processes in which changes in polygalacturonic acid have been implicated, e.g. abscission, should be viewed with caution until it is firmly established that structural changes are correlated with the presence of enzymes.

The emphasis in this study has been on the separation of cultured plant cells. However, the possibility of inducing cell aggregation should not be overlooked. The use of lectins and other macromolecules that bind at the cell surface could provide a different and very informative perspective to the study of surface features that influence aggregate formation. The experimental reaggregation of dissociated cells has been a valuable tool in the study of various features of the animal cell surface. Concanavalin A, readily available in pure form, and other lectins apparently attach to specific surface sites, thereby causing cells to adhere to one another. Cell adhesion is inhibited if the binding sites are competitively occupied
by saccharides which do not participate in the formation of intercellular bonds. Furthermore, lectins are rather specifically displaced by the presence of simple sugars.

The failure in this study to induce spontaneous adhesion of rose cells with concanavalin A does not negate the possibility that lectins may be used to aggregate suspended plant cells. There are many of these compounds and they are known to vary in their specificity (Sharon and Lis, 1972). Concanavalin A, for example, shows a certain preferential binding to mannose, which is present in only trace amounts in cultured rose cell walls. Another lectin, ricin, binds specifically to galactose (Sharon and Lis, 1972). Since cell surface changes involving galactose may be important in the development of rose cell aggregates in culture, it would be particularly appropriate to use ricin in future attempts to induce spontaneous aggregate formation. The ability to induce and regulate spontaneous adhesion of dissociated plant cells would not only facilitate the study of their surfaces; this "tissue reconstruction" could also be used to explore the significance of intercellular contact areas in plant cell communication.
SUMMARY

The results of this investigation are primarily of a descriptive nature. As such, they are not subject to detailed interpretation, but do provide a basic understanding of rose suspension culture development and an indication that cell wall metabolism plays an important role in aggregate formation and dissociation. The principle findings are summarized in the following points.

1. The degree of cell aggregation in suspension cultures of Paul's Scarlet rose is regulated by some type of developmental control. This control manifests itself differently during the various stages of the culture cycle and can be perturbed by modifying the culture medium.

2. Cell aggregate formation, cell separation, and aggregate dissociation are basically different processes. Normally, rapidly dividing cells form large aggregates, but under certain conditions they show a much reduced tendency to adhere to one another. Aggregate dissociation is largely restricted to the stationary phase of growth. Cell expansion may be an integral part of aggregate dissociation but not the separation of proliferating cells.

3. Changes in the amount of galactose in the wall modify the surfaces of rose cells in a way that influences their participation in aggregate formation and dissociation.

4. The decrease in wall galactose associated with aggregate
dissociation may be mediated by cell surface β-galactosidase activity.
LITERATURE CITED


ACKNOWLEDGEMENTS

This project was supported by the Graduate College and the Department of Botany and Plant Pathology; I would like to thank Dr. Frederick G. Smith for making the necessary funds available.

I am deeply grateful to Dr. Donald Nevins. He suggested this problem, and his ideas, encouragement, guidance, and concern have been given most generously during the past three years. Working in his lab has been a privilege and a pleasure.

I have also learned a great deal from Dr. Clifford LaMotte, Dr. Wayne Loescher, and Dr. Cecil Stewart and take this opportunity to thank each of them. Dr. Stewart also kindly provided the original callus of the rose tissue used in this study.

I thank Dr. Harry T. Horner for his instruction in photomicroscopy, and Diane Currie for developing the Omnitab program to handle the gas chromatograph data.

I express a very special thank you to my wife, Barbara, who has helped in so many ways. She developed and printed all photographs and did all the typing (twice). Her love, cheerfulness, and patience have made things easy.