FORMATION AND DISSOCIATION OF CELL AGGREGATES IN SUSPENSION CULTURES OF PAUL'S SCARLET ROSE

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ABSTRACT

In liquid culture stem tissue of Paul's Scarlet rose produces a suspension containing cell aggregates of extremely variable dimensions. There is, however, a definite pattern of change in the degree of cell aggregation over time. During the period of most rapid cell division large aggregates form as the result of a minimal separation of the proliferating cells. As the rate of cell division slows, the average number of cells per aggregate decreases. The dissociation of cell aggregates continues at a uniform rate after cell division has stopped. Cell separation is inhibited at low (0.1 mg/l) auxin (NAA) concentrations and by substitution of sucrose for glucose in the culture medium. Cell separation is delayed (but not greatly inhibited) by kinetin. The presence of casein hydrolysate prevents the formation of the large cell aggregates normally occurring in the early stages of the culture cycle. A variant strain which shows a much higher degree of cell separation has been isolated from stock callus tissue grown on solid medium.

INVESTIGATIONS of many aspects of cell physiology and metabolism have been facilitated by the use of liquid suspension cultures of higher plant tissue. Examples include detailed studies of cell division (Stuart and Street, 1969), cell wall synthesis (Rubery and Northcote, 1970), protein-amino acid biosynthesis (Dougall and Fulton, 1967), and acetate metabolism (Fletcher and Beevers, 1970).

One of the problems encountered in the suspension culture of higher plant cells is the formation of nonuniform tissue clumps that range in size from a few to hundreds and even thousands of cells. Although single cells do occur they are usually released from multicellular aggregates (Street, Henshaw, and Buiatti, 1965). A suspension containing only single cells and cell pairs, i.e., the complete separation of cells following division, has not been reported. The heterogeneity of suspension cultures results in varying surface to volume ratios, unknown diffusion gradients, and other undefined cell contact phenomena. The inability to culture higher plant cells like microorganisms (completely separated from one another) has severely limited the use of liquid culture techniques in genetic and biochemical studies of the type that have become classic in microbiology.

A thorough understanding of how cell aggregates form and dissociate may lead to successful efforts to prevent their occurrence or limit their size. Street et al. (1965) have suggested that experimental control of the natural cementing substances between higher plant cells could be used to limit the size of cell aggregates. Regardless of whether or not such a specific mechanism exists it seems likely that cell aggregation and separation are controlled by events occurring at the cell surface and would likely involve the cell wall.

Our ultimate goal is to examine the role that the cell wall and cell wall metabolism play in the formation and dissociation of cell aggregates. However, to examine the mechanisms of aggregation and separation it must be demonstrated that these processes are subject to some degree of regulation. In this paper we have investigated the growth of Paul's Scarlet rose tissue in suspension culture and report modifications in the medium that affect cell aggregation.

MATERIALS AND METHODS—Paul's Scarlet rose tissue grows well on a completely defined medium and is suited for liquid culture since it can be accurately transferred by pipette. The medium used was a modification of the PN-25 medium described by Tulecke, Taggart, and Colavito (1965); all components of the various experimental media were autoclaved together. The standard carbon source for most stock cultures was 2 % glucose in place of sucrose. Callus cultures were maintained on medium solidified with 0.75 % agar and were subcultured every 30 days. Under these conditions the callus tissue is parenchymatous; it contains no recognizable differentiated cells. Cell suspensions were initiated by transferring 1.5–2.0 g of callus tissue (3–4 wk old) to 500 ml Erlenmeyer flasks containing 125 ml of liquid medium. After 9 days,
TABLE 1. Growth of rose cell suspensions during an 18-day culture period

<table>
<thead>
<tr>
<th>Age: Days in culture</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>Cells per ml</td>
<td>4,225</td>
<td>12,463</td>
<td>44,079</td>
<td>84,488</td>
<td>106,417</td>
<td>104,667</td>
<td>102,583</td>
</tr>
<tr>
<td>Aggregates per ml</td>
<td>40</td>
<td>54</td>
<td>160</td>
<td>365</td>
<td>820</td>
<td>1,200</td>
<td>1,640</td>
</tr>
<tr>
<td>Cells per aggregate</td>
<td>106</td>
<td>231</td>
<td>275</td>
<td>230</td>
<td>130</td>
<td>87</td>
<td>63</td>
</tr>
<tr>
<td>PCV (ml) per 10⁶ cells</td>
<td>6.0</td>
<td>3.8</td>
<td>3.2</td>
<td>3.6</td>
<td>4.7</td>
<td>5.2</td>
<td>6.0</td>
</tr>
</tbody>
</table>

a Cells cultured in control medium containing 2 % glucose, 1.0 mg/l naphthalene acetic acid (NAA), and 0.5 mg/l kinetin.

b Values represent the average of three separate experiments.

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 operated at a rate of 100 excursions per min.

In each experiment cells were cultured in 125 ml Erlenmeyer flasks containing 30 ml medium. Experimental media were inoculated with cells from 13-day-old stock cultures in passage 2, 3, or 4. Inoculum suspensions were prepared by pouring off the spent medium and resuspending the cells in 250 ml fresh medium. This was done to minimize the transfer of components of the conditioned medium. Three ml of this suspension were used to inoculate each flask. This resulted in an initial cell density of 4000-5000 cells per ml in 40–50 aggregates. There were three replicate cultures per treatment.

In most of the relatively few studies of this type, various sieving methods have been used to measure cell aggregation (Torrey and Reinert, 1961; Sussex and Clutter, 1967; Eriksson, 1965; 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 (Liau and Boll, 1971; Henshaw et al., 1966) 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. The average number of cells per aggregate is a more meaningful expression and has been used here. The number of aggregates per ml was determined with a Sedgwick-Rafter Counting Cell. Single cells were counted as one aggregate. An aliquot of the suspension was then treated with a solution of 0.2 % Pectinase (w/v) (Sigma Chemical Co.) in 50 mM sodium citrate buffer (pH 4.6) for 1–2 hr followed by agitation with a vortex mixer for 30 seconds. After appropriate dilutions, cells were counted with the Sedgwick-Rafter cell. The average number of cells per aggregate was calculated from these two counts. Each count was the average of ten microscope fields in each of four 1-ml aliquots. The three replicate cultures of each treatment were combined and diluted to facilitate counting.

Dry weight was determined by freeze-drying cells after separating them from the medium by sieving through Miracloth (Calbiochem).

An estimate of cell size was obtained by centrifuging a sample of the suspension for 5 min (ca. 2200 x g) in a graduated centrifuge tube and calculating the packed cell volume (PCV) per 10⁶ cells.

RESULTS AND DISCUSSION—Changes in cell number, aggregate number, average number per aggregate, and size (PCV/10⁶ cell) during an 18-day culture period are illustrated in Table 1. Photographs taken at 5 and 14 days are shown in Fig. 1. The early stages of the culture cycle are characterized by decreasing cell size and the formation of large aggregates. Cell aggregation reaches a maximum and cell size a minimum by day eight. At about day ten to day twelve cell separation and enlargement become the most conspicuous features of the suspension.

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. These changes may be the result of one or both of two types of cell separation. That is, newly formed cells may separate immediately following division (fail to adhere) and/or aggregates may begin to dissociate into smaller units. After 12 days in culture, division has essentially ceased.
but increase in aggregate number continues at a high rate. The decrease in cells per aggregate after day 12 is apparently due to the fragmentation of aggregates formed during the phase of rapid division.

A comparison of cell size (PCV) and number per aggregate as a function of time suggests that enlargement and separation may be related. Microscopic examination of the rose suspensions revealed that enlarged cells most often occur at the periphery of aggregates, in loose aggregates that contain intercellular spaces, or as filamentous outgrowths of elongated cells (Fig. 1). Free cells are usually much larger than those of the large compact aggregates. This apparent correlation between size and separation is consistent with the findings of Digby and Wareing (1966). Lamport (1964) implies that wall extension throughout the cell promotes separation, and Henshaw et al. (1966) suggest that separation is dependent upon expansion. However, Liau and Boll (1971) express doubt that expansion alone causes cells to separate and suggest that an enzymatic dissolution of intercellular bonds may be involved.

Aggregate size in many types of cultures is sensitive to changes in the medium. The manipulation of culture media has been the usual approach in attempts to obtain plant suspension cultures showing a high degree of cell separation. Our interest in this approach has been in attempts to define conditions which inhibit or enhance separation. Such information would be essential in subsequent investigations of the involvement of the cell wall in aggregate development.

The influence of various carbon sources on growth and aggregate size is shown in Table 2. Other carbon sources were tested in preliminary experiments but supported little or no growth and were not used in studies of aggregate development. These included fructose, mannose, arabinose, xylose, ribose, rhamnose, sorbose, maltose, lactose, and melibiose. Galactose, raffinose, or sucrose in the medium results in the formation of the largest cell aggregates (Table 2). Galactose, cellobiose, and raffinose greatly reduce cell division and dry weight accumulation. Comparisons of aggregate development are subject to question if large differences in growth between treatments exist. Therefore, sucrose was the only carbon source studied in time-course experiments for comparison with the influence of glucose. It was found that the number of cells per aggregate reaches a maximum of slightly over 400 in the sucrose medium and 300 in the glucose medium (Fig. 2). This appears to be due, in part, to a greater rate of division in sucrose cultures during the early stages since aggregate number is the same in both cases up to day eight. 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

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Dry wt. per culture (mg)</th>
<th>Cell number per ml</th>
<th>Aggregate size (cells per aggregate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose&lt;sup&gt;c&lt;/sup&gt;</td>
<td>261</td>
<td>103,800</td>
<td>100</td>
</tr>
<tr>
<td>galactose</td>
<td>42</td>
<td>25,019</td>
<td>197</td>
</tr>
<tr>
<td>cellobiose</td>
<td>115</td>
<td>36,442</td>
<td>138</td>
</tr>
<tr>
<td>sucrose</td>
<td>240</td>
<td>89,972</td>
<td>191</td>
</tr>
<tr>
<td>raffinose</td>
<td>92</td>
<td>52,117</td>
<td>247</td>
</tr>
</tbody>
</table>

<sup>a</sup> All measurements taken after 15-days growth.
<sup>b</sup> Each sugar was used at 20 g/l in the basal medium.
<sup>c</sup> Control.
day 18 the average number of cells per aggregate is three times as great in sucrose despite the fact that the total number is considerably lower. This inhibition of separation during the later stages of the culture cycle is the most striking result of sucrose in the culture medium.

In view of the effect of various sugars on the degree of aggregation, it is of interest to point out that Nevins, English, and Albersheim (1967) found that cell wall composition in sycamore suspension cultures is influenced by the nature of the carbon source. Furthermore, Cox and Gesner (1965) demonstrated that specific sugars in the medium interfere with aggregation in certain mammalian tissue cultures, perhaps by binding complementary sites on the cell surface. This led Nevins et al. (1967) to suggest that sugars in the medium might affect the plant cell's participation in surface interactions by inducing change in the polysaccharides of the surface. The results reported here demonstrate that the carbon source does influence the surface interactions involved in aggregate development.

The possibility that the osmotic difference between the media containing glucose and sucrose might affect cell growth must also be considered. However, it seems unlikely that higher osmotic levels lead to greater separation since cells form very large aggregates in the presence of galactose and, conversely, show a relatively high degree of separation with cellobiose (Table 2).

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 naphthalene acetic acid (NAA). As shown in Fig. 3, 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 the final aggregate size but does appear to slightly enhance separation during the phase of rapid division (Fig. 3). Aggregate number is drastically reduced in 0.1 mg/l NAA although the number of cells produced is only slightly lower than in 1.0 mg/l NAA. A similar promotion of separation by auxin has been reported in suspension cultures of other plant tissues by 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 wall changes; these changes perhaps also are involved in separation.

An inverse relationship between kinetin concentration and the degree of cell separation was observed at day 12. Kinetin was tested at 0, 0.1, 0.5, 5.0, and 10.0 mg/l; the largest cell aggregates occur in high kinetin media. This effect has also been noted in suspension cultures of Haplopappus (Eriksson, 1965) and Acer pseu-

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Fig. 2-5. Effect of various medium components on cell number, aggregate number, and aggregate size. 2. Effect of 2% sucrose (open circles) in place of 2% glucose (solid circles). 3. Effect of naphthalene acetic acid (NAA) at 0.1 mg/l (open circles), 1.0 mg/l (solid circles), and 5.0 mg/l (triangles). 4. Effect of kinetin at 0 mg/l (open circles), 0.5 mg/l (solid circles), and 10.0 mg/l (triangles). 5. Effect of 3/2 x nitrate plus 500 mg/l casamino acids (Difco) (open circles).
strain B and has been subcultured 18 times at 30-day intervals without changing appearance or identity. 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 (Fig. 6).

The striking feature of strain B is its uniform appearance in liquid culture. The increase in aggregate number follows a time course that is very similar to the increase in cell number (Fig. 6). As a result the average number of cells per aggregate remains very low.

In this paper we have described time course changes in the development of cell aggregates in rose suspension cultures. The formation and dissociation of aggregates appear to be temporally controlled and can be regulated by altering the nutrient medium. Aggregate size can be maintained at approximately 40 cells per aggregate during the entire culture cycle by the addition of casein hydrolysate to the medium. At the other extreme, the large aggregates that normally develop can be made to persist by low auxin or the presence of sucrose after cell division ceases. Although the size of aggregates that can be predictably produced includes a wide range (40–400 cells per aggregate) this approach seems to be of limited usefulness in attempts to obtain true single-cell suspensions. Simple modifications of the culture medium appear not likely to result in complete cell separation in typical suspension cultures. However, the ability to control aggregate size should make possible attempts to correlate changes in the degree of separation with biochemical modifications of the cell surface.

**LITERATURE CITED**


Street, H. E., H. A. Collin, K. Short, AND I. Simpkins. 1969. Hormonal control of cell division and ex-


