QUANTITATIVE ELECTRONIC ANALYSIS OF NORMAL AND TRANSFORMED BHK21 FIBROBLAST AGGREGATION

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SUMMARY
The kinetics of cell aggregation in shaking suspension are complex. Consequently most existing forms of assessment are qualitative or semiquantitative. We have used a Coulter counter coupled to a particle size discriminator to describe aggregation more precisely.

BHK21 C13 cells were suspended by light or heavy trypsin/EDTA treatments, and then aggregated at 37 °C. Plots were obtained showing the distribution of cells in 1–50 cell aggregates, from which were calculated the rates of redistribution of cells between aggregates. About half the cells in the initial suspension were not single. During the first 5 min of aggregation single cells and aggregates containing up to 4 cells disappeared into larger aggregates. At later times there was a shift towards a net loss of slightly larger aggregates. Adhesions between single cells were the most common event throughout aggregation, but they declined relatively rapidly with time as adhesions between aggregates became relatively more prominent. The overall rate of adhesions declined 10-fold within 12 min and 100-fold within 90 min.

In contrast to normal fibroblasts, nearly all transformed BHK21 cells in initial suspensions were single, and even by 30 min few single cells had adhered to form aggregates. The initial rate at which adhesions were formed was only about 15 % of that of normal cells.

Heavy trypsin/EDTA treatment of normal cells released DNAse-sensitive material which markedly altered the aggregation kinetics. These changes included the disaggregation of small loosely adhering cell clusters coupled with the formation of abnormally large aggregates.

It is suggested that careful preparation of suspensions together with assessment of aggregation on the basis of initial adhesion rate will improve the accuracy and information yield in this type of experiment.

INTRODUCTION
Since first being described by Moscona (1961) the aggregation of cells in shaking suspensions has been widely used as a means of investigating the strengths and specificities of intercellular adhesions and the agglutination of cells by plant lectins. Because the parameters which determine the rate or degree of aggregation in any suspension of particles are constantly changing as adhesions between particles occur, the kinetics of aggregation and agglutination are complex, thus making quantification difficult. In some circumstances aggregation may finally result in a fixed number of aggregates of similar size; however, even when this can be achieved Curtis (1973) has questioned the validity of attempting to quantify aggregation on

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this basis. Usually, aggregation is assessed at one or more intervals, when samples contain unknown proportions of single cells and aggregates of different sizes. In theory complete characterization can be achieved by direct visual analysis, but in practice the observer cannot accurately detect the number of cells in aggregates containing more than about 5 cells (Curtis & Greaves, 1965; Edwards & Campbell, 1971; Waddell, Robson & Edwards, 1974). Although most forms of electronic particle counters are technically capable of yielding a full analysis of particle size and frequency distribution they are rarely used in this way. This is because such analyses require very large numbers of cells and involve repeated counts over a wide range of settings. Consequently these counters are most commonly used to monitor such simple parameters as single cell or total particle counts, or a combination of the two (Lilien, 1968; Orr & Roseman, 1969a; Vicker & Edwards, 1972; Deman & Bruyneel, 1973). There has been no convincing evaluation of the correlation between changes in these parameters and the overall process of aggregation, primarily because not enough is known about the latter. This paper examines these problems and identifies some potential common sources of error in aggregation experiments, as well as recommending a form of aggregation assessment which measures the adhesiveness of the cells involved.

It becomes possible to analyse the distribution of cells in aggregates over a wide range of sizes by coupling a particle counter with a 64-channel analyser for automatic sorting and storing of particle size and frequency data which has a capacity to count large samples of cells. We have used this technique on BHK21 C13 fibroblasts and their polyoma-transformed derivatives treated lightly and heavily with trypsin/EDTA to deduce within certain limits the distribution of cells and the rates of redistribution at different stages of the aggregation process, and have also investigated a number of practical problems which occur in experiments of this type. Our findings demonstrate that misleading results can be obtained not only when changes in single cell or total particle counts are used to assess aggregation, but also when the aggregation of different cell types is compared. We also demonstrate that the procedures used to produce the initial cell suspension can alter the aggregation characteristics fundamentally.

MATERIALS AND METHODS

BHK21 C13 fibroblasts and polyoma virus-transformed BHK21 Py6 cells were obtained from the Imperial Cancer Research Fund, London. They were grown in Eagle’s minimal essential medium with 10% foetal calf serum, and were harvested from glass when subconfluent and still in exponential growth. Cells were resuspended by 2 methods called light and heavy trypsin/EDTA treatments. Light trypsin/EDTA treatment entailed incubating the cells at room temperature in 3 changes of Ca2+ and Mg2+-free phosphate-buffered saline (PBS) for a total of 30 min followed by immersion in the same solution containing 0.0025% trypsin (10800 BAEE units per mg, Sigma, type III, St Louis, Mo., U.S.A.) and 0.016% EDTA for a few seconds until the cells started to detach under vigorous agitation. This medium was then immediately replaced by ice-cold Ca2+- and Mg2+-free PBS propelled through a syringe to suspend the cells. Three washes were then carried out in the same medium by centrifugation for 10 min at 1000 rev/min at 4 °C in order to prevent residual trypsin or EDTA being carried over to the aggregation medium, and the pelleted cells were
resuspended in cold unsupplemented Eagle's medium by vigorous agitation followed by two passages through a 26-gauge hypodermic needle. Heavy trypsin/EDTA treatment entailed only one short rinse in Ca²⁺- and Mg²⁺-free PBS followed by immersing the cells in the same medium containing 0.5% trypsin and 0.2% EDTA until they started to be detachable by agitation. The medium was then poured off and the cells left at room temperature for 10 min in the residual medium. They were then collected and washed as for light trypsin/EDTA treatment. Initial viability of cells prepared by either method as judged by trypan blue dye exclusion was better than 90% in all experiments. There appeared to be no decrease in cell viability as aggregation progressed, and dead cells were randomly incorporated into aggregates.

Aggregation

Particle concentration was adjusted to 1 × 10⁶/ml and 2–5-ml aliquots were transferred to twice-siliconized stoppered 25-ml conical flasks gassed with 5% CO₂ in air. Flasks were warmed to 37 °C for 10 min before aggregation, which was carried out at 37 °C by orbital shaking at 100 rev/min for between 5 min and 2 h. The suspension was then diluted 1:50 with Isoton (Coulter Electronics, Harpenden, U.K.) to give maximum particle concentrations for counting of less than 2 × 10⁴/ml. Size distribution plots were obtained using a model FN particle counter coupled to a model P64 channel analyser (Coulter Electronics), an XY plotter and oscilloscope. The counter settings were; orifice 140 μm, aperture 512, and size distributions were obtained from alternate attenuation settings between 4 and 0.125. The largest aggregates were counted first in order to minimize the effect of sedimentation. The plot was made with the edit facility on, except during calibration against latex particles, and the threshold, calibrated to conform to that of the counter, set at 20. Large infrequent aggregates were sought for 100 s at the lowest count range setting; this allowed single large aggregates present at an average frequency of not less than 1 per 160 000 cells to be detected. Smaller, more frequent aggregates were detected initially by increasing the count range and subsequently by reducing the counting time. Each plot was monitored on the oscilloscope and the range and time adjusted accordingly. Each plot was of gradually decreasing width; the first covering aggregates from 20 to 50 cells in size, the last covering single cells only. The data from plots were pooled and the numbers of cells present in aggregates of different sizes were calculated after determining the volumes of the cells by calibration against latex spheres of known size. This procedure allows the P64 plots to be calibrated against the known mean volume of the spheres, but it is not then possible to determine accurately the mean size of single cells under examination since there is no discontinuity between the sizes of the largest single cells and the smallest 2-cell aggregates. We have therefore elected to use the mode volume since this can be determined accurately for single cells. We have, however, investigated the relationship between mean and mode volumes by visually measuring the diameters of 500 of each cell type used. We found that the mode volumes of BHK21 C13 and Py6 cells were 95 and 117% of the mean volume, respectively; thus mean and mode volumes are fairly similar to each other.

The procedure for determining cell number per aggregate, described above, assumes a linear relationship between recorded particle volume and the number of cells per particle. Some difficulty was experienced in incorporating data on large aggregates since this information tended to be incomplete to the extent that not all sizes of large aggregates were represented or present in sufficient quantities to yield meaningful continuous plots. Consequently although individual large aggregates were readily detected by this technique they do not appear in the figures, which have been trimmed to eliminate the 1% of total cell population present in the largest sizes of aggregates by plotting their log values. The rates at which events occurred during aggregation are expressed in numbers of cell-cell adhesions per hour. Since the cell concentration in the 1–50 cell aggregate window changed during aggregation the total number of cells present in the window at any time was first converted to 100, and the concentration of cells in any one size of aggregate was then expressed as a percentage of this total. When 2 cells adhere it is clear that a single adhesion has formed; however, the situation is more complex when an aggregate adheres to a single cell or other aggregate. Nevertheless we have opted to express adhesion on the basis of half
Fig. 1A–D. Light micrographs of BHK21 C13 fibroblasts resuspended from monolayer by light trypsin/EDTA treatment and subsequently allowed to aggregate in serum-free medium in a shaking incubator at 100 rev/min and 37 °C. ×120. 

A, initial cell suspension prior to aggregation, in which 46% of the cells were single and the rest present in small aggregates. B, after 5 min incubation. Examples of the small aggregates having an elongated form and therefore likely to be eliminated electronically from particle counts are marked with arrows. C, after 30 min incubation. D, after 2 h incubation.
the total number of cells involved, as there appears to be no more satisfactory method available. Thus the number of adhesions formed has been calculated as half the sum of the negative rates.

RESULTS

The data obtained have been expressed in 2 ways; the first shows the distribution of cells in aggregates of different sizes at different times during the aggregation process (Figs. 2, 6, 8); the second, the rates at which net redistributions of cells between aggregates occur (Figs. 3, 7, 9). These values express only the net flow rates, first because most sizes of aggregate are being formed at one rate by adhesions between single cells and/or smaller aggregates and are being lost at another as they themselves adhere to single cells or aggregates, and secondly because we do not know to what extent the aggregation process involves a simultaneous process of disaggregation.

Aggregation of BHK21 C13 fibroblasts lightly treated with trypsin/EDTA

The distribution of cells in aggregates of different sizes at various stages of the aggregation process is shown in Fig. 1. When analysed on the counter (Fig. 2) the initial ‘single’ cell suspension contained only 44% of single cells (mode volume 1215 μm³, 13.2 μm spherical diameter), the remainder being distributed in small

![Fig. 2. Aggregation of BHK21 C13 fibroblasts in shaking suspension in serum-free medium at 37 °C, after removal from monolayer culture by light trypsin/EDTA treatment. The distribution of cells in aggregates of different sizes in the initial cell suspension (■) and after 5 (▲) and 30 min (●) of incubation has been plotted semi-logarithmically to exclude aggregates containing less than 1% of the total cell population.](image-url)
aggregates. This compared favourably with a value of 46% obtained by visual analysis of the suspension shown in Fig. 1A. After 5 min of shaking incubation (Figs. 1B, 2) the single cell population had declined and there were lesser decreases in the number of aggregates containing 4 or less cells, while aggregates containing 5 or more cells increased in number. The number of cells lost by the disappearance of single cells and small aggregates was not compensated for by their reappearance in larger aggregates since by 5 min the total number of cells present in the 1–50 cell aggregate window had declined to 79% of the original number. The redistribution of cells between 5 and 30 min (Figs. 1B, C, 2) resembled that occurring between 0 and 5 min, but there were differences of degree; for example single cells declined much less markedly, and larger aggregates, containing up to 19 cells, were formed (aggregates containing up to 50 cells were detected in all these experiments but the extreme rarity of those containing more than about 25 cells made it impossible to determine their rate of formation with a reasonable degree of accuracy). There was a further reduction in the total number of cells in the 1–50 cell aggregate window, to 60% of that in the initial cell suspension. Only marginal differences were observed

Fig. 3. Aggregation kinetics of light trypsin/EDTA-treated BHK21 C13 fibroblasts showing the net rates of formation or loss of different sizes of aggregate. The data have been obtained by determining the net loss or gain of cells for each size of aggregate during the first 5 min (■) and between 5 and 30 min (▲) of shaking incubation. These values have then been adjusted to give the net loss or gain per h expressed semilogarithmically as a percentage of the average total cell concentration during the period of aggregation in question. Cell movements to or from aggregates of less than 1% per h have been excluded.

of cells between 5 and 30 min (Figs. 1B, C, 2) resembled that occurring between 0 and 5 min, but there were differences of degree; for example single cells declined much less markedly, and larger aggregates, containing up to 19 cells, were formed (aggregates containing up to 50 cells were detected in all these experiments but the extreme rarity of those containing more than about 25 cells made it impossible to determine their rate of formation with a reasonable degree of accuracy). There was a further reduction in the total number of cells in the 1–50 cell aggregate window, to 60% of that in the initial cell suspension. Only marginal differences were observed
after 2 h of incubation (Fig. 1D); consequently observations made at this time will not generally be described.

For the first 5 min single cell adhesions (either to single cells or to aggregates) were the most common event (Fig. 3); these dropped almost twelvefold between 0 and 5 min and 30 min and 2 h. In contrast the rate of loss of small aggregates (4 cells or fewer) slowed much less rapidly; thus aggregation initially occurred primarily by the loss of single cells into aggregates, but gradually aggregate-aggregate adhesions contributed an increasingly significant proportion of the changes observed. The

![Graph showing comparison of aggregation rates for normal (■) and transformed (▲) BHK fibroblasts at different times of incubation.](image)

Fig. 4. Comparison of the rates of aggregation of normal (■) and transformed (▲) BHK fibroblasts at different times of incubation. The rates have been obtained by halving the total individual negative rates of cell redistribution to each size of aggregate containing up to 20 cells, and the time of each reading has been fixed as the log of the mean time between which the 2 sets of readings were made.

overall movement was an initial shift from single cells through small aggregates to the gradual formation of increasingly large aggregates from smaller ones as aggregation progressed. Another major feature was the rapid decline in rate of aggregation, evidenced by the reduction in rate of formation of new adhesions (Fig. 4), and presumably due to the decline in particle concentration rather than to any change in collision efficiency.

**Aggregation of cells heavily treated with trypsin/EDTA**

This treatment resulted in aggregation kinetics markedly different from those of lightly treated cells. The main feature was the rapid development of a few large aggregates containing many thousands of cells (Fig. 5) and visible to the naked eye. Addition of crude DNase (Sigma) at 500 Kunitz units/ml before the onset of
aggregation totally inhibited the formation of these large visible aggregates, and altered the aggregation kinetics to the extent that more single cells and smaller aggregates were found in DNase-treated than untreated solutions after 1 h of incubation. Thus aggregation of such cells was at least partly due to leakage of DNA or DNA-associated material. Fig. 6 shows the aggregation kinetics of heavily trypsin/EDTA-treated cells in the absence of DNase. The initial cell suspension contained only 18% of single cells; far fewer than in the lightly trypsin/EDTA-

![Image](image_url)

Fig. 5. Aggregation of BHK21 C13 fibroblasts recovered from monolayer culture by heavy trypsin/EDTA treatment. The aggregate, which was formed by 5 min of shaking incubation has a volume of about 1.9 mm³ and therefore contains an estimated 1.5 x 10⁹ cells. Such aggregates did not form in the presence of DNase at 500 Kunitz units/ml. x 31.

treated samples. The majority of cells were present in aggregates which were predominantly of small size. The changes in cell distribution taking place during the first 5 min of aggregation were almost the reverse of those seen after light treatment; the number of single cells more than doubled while there was a reduction in the numbers of aggregates of all sizes. Concomitant with these changes was the build-up of a few very large aggregates (Fig. 5) which could not pass through the orifice of the particle counter. Between 5 and 30 min the increase in the number of single cells and the decline in the numbers of aggregates continued. The rate diagram (Fig. 7) shows that the major early feature was the disintegration of small aggregates into single cells. Between 5 and 30 min the build-up of single cells had slowed down to almost zero; however, since small aggregates continued to disappear this must have been due mainly to their incorporation into the very large DNase-sensitive aggregates.
Aggregation of polyoma-transformed fibroblasts

The number of single cells (mode volume 898 μm³, 12.0 μm spherical diameter) present in the initial cell suspension, 92% (Fig. 8), was considerably higher than that of normal BHK21 C13 fibroblasts. Almost all of the remainder were present in 2–3 cell aggregates, and the largest aggregate ever detected in an initial suspension contained 8 cells. After 5 min of aggregation the total number of detectable cells had dropped to 83% of that in the initial cell suspension; 88% of these were still present as single cells, the remainder being present in very small aggregates (Fig. 8). After 30 min of aggregation this distribution had again changed relatively little; 83% of the total cell population (now reduced to 57% of the initial cell concentration) was still present as non-aggregated single cells, but the number of small aggregates had increased slightly, particularly of the 2-cell aggregates which now accounted for 13% of all cells. Between 0 and 5 min the rate of loss of single cells (Fig. 9) was about one quarter that of normal BHK cells; in contrast to normal BHK cells the rate of loss of single cells declined only 3-fold between 0 and 5 and 5 and 30 min. The formation rate of aggregates was very slow, and declined markedly with increasing size. These observations reflect the lower adhesiveness of polyoma-transformed BHK21 cells, which is initially about one sixth that of the normal cells (Fig. 4).
DISCUSSION

Theoretical aspects of aggregation

Adhesions are generated by collisions which result from particle movement. Since random particle movement (e.g. Brownian movement) cannot be achieved in laboratory systems the favouring of one type of motion (e.g. rotatory) for another (e.g. reciprocal) is meaningless. This comment does not apply to laminar shear flow systems, where a more precise analysis can be conducted under specialized conditions (Curtis, 1970). We have compared (unpublished) the aggregation kinetics of BHK cells in reciprocal and rotary shakers and the results obtained were identical. However, this may not always be the case and it should be accepted that all systems distort random particle movement, and hence aggregation, particularly where there is heterogeneity in particle volume. Because of this latter effect strongly adhesive particle kinetics will be more distorted than those of weakly adhesive particles. Collision frequency is altered only by the changing radius of the particles and their concentration; this is true where gyration approximates random movement and also for shear fields (Smoluchowski, 1917) so it is probably true for the intermediate state generated by laboratory shakers. Unfortunately, although the theoretical and observed relationships between collision frequency and the rate of change of size distributions of particles in hydrosols...
Fig. 8. Aggregation of BHK21 Py6 transformed fibroblasts in shaking suspension at 0, 5 and 30 min (■, △, and ○, respectively). The experimental conditions and presentation of data are the same as described for BHK21 C13 fibroblasts (Fig. 2).

Fig. 9. Aggregation kinetics of light trypsin/EDTA-treated BHK21 Py6 transformed fibroblasts. The data have been obtained in the same way as described for BHK21 C13 fibroblasts (Fig. 3) and show the net redistribution of cells/h between aggregates at 0–5 min and 5–30 min, ■ and △ respectively.
have been analysed in detail (Swift & Friedlander, 1964) the equation contains no function for adhesiveness. Fuchs (1934) expanded the original theoretical analysis of Smoluchowski (1917) to include a term for the interaction of particles but since this applies only to physical repulsions and attractions it may not be applicable to the present study. One further aspect, however, may be significant; Swift & Friedlander (1964) show theoretically and experimentally that the size distributions of coagulating hydrosols tend to preserve their shape. It is possible therefore that initial particle shape and size, which are different in normal and transformed cell populations, may tend to be conserved during aggregation. Thus cell populations of widely different morphologies may have built-in differences in their aggregation kinetics which might erroneously be attributed to different adhesiveness.

Assessment of the technique used

We have provided quantitative data to describe the aggregation kinetics of normal and polyoma-transformed BHK21 fibroblasts in shaking suspensions, based on changes in size-distribution plots showing the net redistribution of cells between single cells and aggregates of various sizes. These data were obtained from an electronic particle counter and size discriminator which consigned them to 64 memories on the basis of particle size. A number of practical and theoretical considerations limit the range and accuracy of the technique. For example the height of pulse produced by a particle passing through the orifice of the counter is affected by the relationship between the relative sizes of the particle and the orifice. Because of this, using a 140-μm orifice, only pulses from particles of volumes less than 50,000 μm³ were acceptable. In addition, a lower threshold of 465 μm³ was used to eliminate electronic background noise. This effectively limited our investigations of aggregation to a range extending from cells of about half the mode volume of exponentially growing normal BHK21 C13 fibroblasts up to aggregates containing a maximum of 50 cells. The counting and sizing accuracy of samples containing such a wide range of different sizes are impaired because of the variable effects of particles of different sizes on horizontal and vertical coincidence interactions as well as pulse distortion due to non-axial flow resulting from the proximity of particles to the edge of the orifice (Shulz & Thom, 1973). This problem was minimized in 2 ways. First, the overall size distribution was collected on 6 plots of minimum window width (51 channels) with gradually decreasing attenuation settings for smaller aggregates. Secondly, the edit facility of the channel analyser was used to select only those pulses which conformed to certain preset pulse height-to-width ratio limits, as a result of which up to 50% of pulses were eliminated. These misshapen pulses comprised primarily horizontally or vertically coincident small particles, particles of any size moving through the orifice at its edge, and large particles passing close to, but not through, the orifice, since the sensing zone extends out for 60-70 μm in front of the orifice. A further complication detected by visual observation of aggregating samples was that cell chain conformations were frequent amongst aggregates containing 2-4 cells (Fig. 1B). The resulting deviation of such elongated aggregates from the normal pulse height-to-width ratios would lead to their selective
elimination when compared to more nearly spherical single cells or large aggregates. Unfortunately we have no means at present to quantify this potential source of bias; nevertheless it is apparent that the accuracy of the size distribution curves plotted is probably not uniform for aggregates of different sizes. Consequently, while changes in the proportion of cells present in aggregates of any particular size may be convincingly recorded by this technique provided the concentration of cells is kept low, analysis of the redistribution of cells between aggregates of different sizes may be subject to some degree of distortion. Nevertheless these results represent a substantial advance in the detail and consequent accuracy of analysis of the events occurring during the aggregation process over all methods currently available, except those incorporating hydrodynamic focusing (Shulz & Thom, 1973). As far as we are aware, however, this latter principle has not so far been applied to aggregation analysis.

Initial cell suspensions

In the case of normal BHK21 fibroblasts only 44% of cells were single, compared to 92% of polyoma-transformed cells; a quantitative difference of this type has also been reported by O'Neill & Burnett (1974). Since we have found that this clumping was present even in normal cells resuspended from monolayers at very low density where intercellular contacts were minimal it indicates that normal cells showed a far greater tendency to adhere on contact prior to shaking incubation than did transformed cells. These adhesions presumably occurred early during the preparative procedure since these cells do not aggregate at 4 °C to any extent (Edwards & Campbell, 1971). The differing nature of initial suspensions from cells of different lines raises questions concerning the validity of comparing their subsequent behaviour. For example if the initial dilution of a sample is based, as is usual, on the total particle count, large errors in cell concentration may be introduced so that the aggregation of two samples may be assessed at widely differing concentrations which are not strictly comparable (Oppenheimer & Odencrantz, 1972). We found, for example, that concentrations of BHK21 C13 cells were 3 times greater than Py6 cells diluted to equal concentration on the basis of total particle counts. A further factor requiring consideration in unsynchronized populations is that when single cells form a high proportion of the initial cell suspension (e.g. BHK21 Py6) their adhesive characteristics will be representative of the whole population, but where they form a minority (e.g. BKH21 C13) they may contain a high proportion of an atypical relatively non-adhesive subpopulation such as cells in or near mitosis. Walther, Öhman & Roseman (1973) produced evidence that such a subpopulation of normal BHK21 cells would contain 17% or less of the total cell number; this would account for up to 40% of the single-cell population and would thus have a major influence on single-cell counts. These factors will give rise to an incorrect analysis of adhesiveness in cases where single-cell or total particle counts form part of the assessment technique.
Loss of cells

The total number of cells recorded in samples declined as aggregation proceeded despite the fact that Orr & Roseman (1969b) have reported that the measured volume of cells did not decline during aggregation. In the case of heavy trypsin/EDTA-treated cells, large aggregates were formed which would not have been detected in our 1–50 cell aggregate window and which in some cases could not pass through the orifice of the counter. With light trypsin/EDTA-treated and transformed BHK21 cells, however, we detected only a few aggregates containing more than 20–30 cells. Consequently large aggregate formation could not have been a major cause of cell loss in these 2 cases. On the other hand, normal BHK21 cells were seen to adhere in substantial numbers to the base of the flask, despite the fact that all glassware used was twice siliconized, and this was probably the major cause of loss with these cells. Since polyoma-transformed cell aggregation was typified by small aggregate formation, electronic elimination of elongated particles was probably a particular cause of loss with these cells. Also, some cell death may have occurred during aggregation; Orr & Roseman (1969a) have reported that the Coulter counter fails to register dead cells which have become ‘leaky’. Although we cannot quantify these factors it appears that the causes and extent of cell loss are likely to vary depending upon the cell lines used and their mode of preparation. This phenomenon should be taken into account when aggregation is being assessed, particularly when single-cell or total particle counts are being used to monitor aggregation.

Formation of aggregates

A possible criticism of our data is that conversion of all values to percentages may have masked the fact that no absolute increase in the numbers of any aggregates actually occurred; the apparent increases recorded being due only to a proportional decrease in the numbers of single cells and some smaller aggregates. However, in most cases (95% of light trypsin/EDTA-treated and 100% of heavily treated normal BHK21 cells, and 75% of transformed cells) counts recording a relative increase in the numbers of aggregates also recorded an absolute increase, even prior to correction for cell loss. The formation of aggregates of normal BHK21 cells during shaking suspension was clearly visualized by the redistribution of cells and small aggregates into larger groups. The process of aggregation was most rapid between 0 and 5 min; this contrasts with the report of Walther et al. (1973) that there was a 5-min lag during which no adhesions occurred. Marked differences were observed when the aggregation of normal and transformed cells was compared. Our data show that collisions between Py6 cells less often lead to the formation of adhesions and the production of aggregates than collisions between C13 cells. Compared to normal fibroblasts the disappearance of single Py6 cells occurred at less than one quarter of the rate, despite the fact that the number of single cells was twice as high in the latter and the initial collision frequency was about the same in both suspensions since they contained equal numbers of particles of only slightly different diameters. However, there were also changes in the rate of aggregation in these two cell types.
as aggregation proceeded. Thus while the initial rate of adhesion between normal cells was about 6 times higher than in transformed cells, the rates had become almost the same by 1 h. Since the rate at which adhesions form relative to the number of collisions is a measure of adhesiveness it is apparent that the initial rate of adhesion obtained at a time when conditions in the 2 suspensions are most comparable, is the most accurate method for the assessment of aggregation.

**Effect of trypsin/EDTA treatment**

Trypsin is known to affect the aggregability of cells (Edwards & Campbell, 1971; Walther *et al.* 1973; Deman, Bruyneel & Mareel, 1974; Cassiman & Bernfield, 1975). The heavy trypsin/EDTA procedure we used was designed to be comparable to the more severe treatments likely to be encountered by cells. This not only caused some of the cells to leak DNA but also changed their aggregation kinetics. In contrast to our findings, Edwards & Campbell (1971) reported that DNA-associated material released from lysed cells did not affect the extent of aggregation. This difference may be due to their reliance on single cell or total particle counts to monitor aggregation. The changes we observed included the dissociation of loosely attached aggregates into single cells when incubation commenced, resulting in an increased single cell count. At the same time very large aggregates were formed, so that the increase in single cells was coupled with a decrease in total particle count. Later on all the remaining small components in the system were being gathered up by aggregates now large enough to be visible to the naked eye. The way in which the cells are removed from their substrate therefore has a profound effect upon subsequent aggregation kinetics. We do not know to what extent the aggregation of light trypsin/EDTA-treated cells was altered, but there may not be a well defined level at which no cells are affected in this way. For example, Maizel, Nicolini & Baserga (1975) have reported that 1-min treatment of WI-38 fibroblasts with 0.25% 1:250 trypsin caused a 40% loss of nuclear chromatin-associated proteins. Because of this it is necessary to consider the different susceptibilities of cells to the procedure used to suspend them prior to aggregation. For example, during light treatment we have routinely recorded the number of seconds exposure to the trypsin/EDTA mixture before cells could be detected detaching from the substrate; these averaged about 45 s for normal but only 5 s for transformed cells. Exposing the cells for the same time ignores the possibility that cells may differ in their susceptibility to trypsin/EDTA-mediated damage.

**Use of electronic particle counting for aggregation assessment**

The aggregation kinetics of BHK21 cells may not be typical of other cell lines (Curtis, personal communication, 1976). However, within this limitation it is clear from the results of our analysis of aggregation kinetics that none of the techniques based on the monitoring of single and/or total particle counts provides results which closely reflect the total aggregation process. Furthermore, there are some circumstances in which these methods may actually provide misleading data; for example, we have discussed above a situation in which a shaking cell suspension
actually registered decreased aggregation using single cell counts but increased aggregation using total particle counts. The following factors, derived from our data, should be taken into account when an assessment of aggregation is being made. (a) Initial cell suspensions of different cell lines prepared by the same method may contain different numbers of single cells. (b) Initial cell suspensions may contain different cell concentrations when the total particle count is the same. (c) Initial cell suspensions may exhibit different degrees of aggregation prior to the onset of shaking incubation. (d) Trypsin/EDTA treatment changes aggregation kinetics by causing substantial weak cell-cell adhesions prior to the onset of aggregation, releasing single cells from aggregates as shaking commences, and a rapid build-up of very large aggregates. (e) Different cell lines vary in susceptibility to trypsin/EDTA and may sustain different degrees of damage when treated under the same conditions. (f) The total number of cells may decline as aggregation proceeds; this may be for unrelated reasons in different cell lines. Cell loss is greatest from the single cell population, so that the number of aggregates formed will be less than predicted. (g) Different cell lines aggregate at different rates and the ratio of these changes with time. (h) A decline in single cells is an important feature of the early stages, but not necessarily of the later stages of aggregation. And (i) Aggregation of single cells is a relatively more prominent feature in cell lines of low than of high adhesiveness. Because of these problems we suggest that carefully controlled cell preparative procedures should be coupled with the assessment of aggregation by measurement of the initial rate of adhesion as a means of eliminating errors and optimizing the information obtainable from aggregation experiments.

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Electronic analysis of fibroblast aggregation


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