The measurement of cell adhesiveness
by an absolute method

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The development of a quantitative method for measuring cell adhesion would allow tests to be made of a variety of hypotheses concerning the role of cell adhesiveness in many morphogenetic processes, such as segregation (Steinberg, 1963; Curtis, 1960, 1967), contact inhibition of movement (Abercrombie, 1961), malignancy, etc. Furthermore, the development of a quantitative method giving absolute measurements of cell adhesiveness would be of considerable value in that it would allow critical experiments to be made to test hypotheses about the mechanism of cell adhesion. Basically, two methods exist for the measurement of cellular adhesiveness: (i) a measure of the force or energy required to reseparate two cells or a group of cells from one another or from a non-cellular substrate; (ii) a measure of the forces or energies of interaction involved in bringing two cells or cell groups into adhesion. The first method was introduced as a qualitative test of adhesiveness by Dan (1936). Recently, Brooks, Millar, Seaman & Vassar (1967) have made ingenious calculations of the probable magnitude of the adhesive energies which have to be overcome in dispersing a tissue by mechanical means into single cells. Measurement of the forces that have to be used to disperse the tissue can be used to derive a value of the adhesiveness of the cells as Brooks et al. (1967) have done. But there are difficulties in this method since intercellular materials, such as collagen, may contribute to the average resistance of a tissue to dispersion by mechanical forces, and if the hypothesis advanced by Weiss (1961) is correct, the measurement is not of the adhesiveness of cells but of the mechanical resistance of the cells to being broken. With the first of these considerations chiefly in mind, it seemed desirable to develop a method for measurement of adhesiveness using the formation of adhesions between cells.

A simple process for the formation of adhesions is to reaggregate a suspension of single cells, a technique first introduced by Gerisch (1960). When a suspension of single cells is shaken, they collide and some of the collisions result in the formation of adhesions between cells: in this way, aggregates are built up. Moscona (1961) has suggested that a measure of the adhesiveness of cells is

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given by the diameter of the aggregates. Overbeek's treatment (Albers & Overbeek, 1960) of the limiting size of aggregates might be applied to Moscona's measurements to give an absolute measure of cell adhesiveness, but in practice, aggregate diameters are rather variable for identical experimental conditions, aggregates are not perfectly spherical, and the final aggregate size may be not a simple measure of adhesiveness but a reflection of the changing history of cell adhesiveness during the process.

In this paper, two basic concepts have been used to derive an absolute measure of the adhesiveness from measurements of the rate of reaggregation of cells. The first of these is that, when particles such as cells are brought into collision by shaking the cell suspension, the movements of the medium first bring particles together and then tend to reseparate them, as the particle which travels more rapidly passes the other particle. If the adhesive force is sufficient, the particles may reach a closeness of approach before reseparation starts such that the adhesive force between the particles can prevent the break-up of the newly formed aggregate. The greater the adhesive force, the larger will be the proportion of collisions which will be effective in forming adhesions. This is the second concept to be used in this paper, namely that the proportion of collisions which result in adhesions is a measure of adhesiveness. This proportion, the stability ratio, has been previously used as a measure of particle adhesiveness, but it has rarely been measured directly. Although Fuchs (1934) derived an absolute measure of the adhesive energy between particles from measurement of the stability ratio, there are restrictions in his treatment. First, his treatment presupposes that the particles come into molecular contact, secondly, it is presumed that Brownian motion provides the force that brings two particles close enough for adhesive forces to act and thirdly, a potential energy barrier is assumed to be present tending to prevent collisions leading to adhesion. Since in reaggregation the much larger hydrodynamic shear forces bring particles into adhesion it is obvious that Fuchs's treatment is inapplicable. Moreover, it is probable that biological cells adhere in the secondary minimum, under which conditions the surfaces do not come into molecular contact (Curtis, 1960, 1967). Consequently, Dr L. Hocking and I (A. S. G. Curtis, & L. Hocking in preparation) have developed a new technique for deriving the adhesive force from measurement of the stability ratio, which can be applied whether or not the particles adhere in molecular contact.

METHOD

(a) Theoretical

Although a detailed theoretical description of the method with relevant experimental tests will be published (Curtis & Hocking) it seems appropriate to outline the main features of the theoretical approach here. The essentials of the method are the measurement of the actual number of collisions which
produce adhesions per unit time interval, and the use of a technique which allows calculation of the total number of collisions per unit time. The ratio of the effective collisions to the total number of collisions gives the stability ratio directly. If particles are brought into collision by a laminar shear gradient of shear rate $G$, the total number of collisions per unit time interval $b_{ij}$ can be derived from the relationship

$$b_{ij} = \frac{4}{3} G n_i n_j (r_i + r_j)^2,$$

(1)

where $n_i$ and $n_j$ are the concentrations of particles of radius $r_i$ and $r_j$ per unit volume.

It will be appreciated that once aggregation has started to form two-particle aggregates, these may form either three or four bodied aggregates as a result of collision. Thus equation (1) cannot be applied directly once an appreciable number of two-celled aggregates have formed. Swift & Friedlander (1964) have developed a technique for integrating the total number of collisions for all classes of aggregate size to give the relationship between the total number of particles at time $t$, $N_{\alpha t}$, compared with those at the start of aggregation $N_{\alpha 0}$, the shear rate $G$, and the volume fraction of particles in the suspension $\phi$

$$\ln \frac{N_{\alpha t}}{N_{\alpha 0}} = \frac{-4G\phi t}{\pi}.$$ 

(2)

If only a proportion of collisions are effective in producing aggregates the relationship becomes

$$\ln \frac{N_{\alpha t}}{N_{\alpha 0}} = \frac{-4G\phi\alpha t}{\pi},$$

(3)

where $\alpha$ is the stability ratio (collision efficiency), namely the probability that a collision between two particles results in their adhesion. Consequently, if cells can be aggregated in a laminar system of shear rate $G$ it is directly possible to measure the stability ratio. Use of a Couette viscometer provides an apparatus in which stable known shear rates ($G$) in laminar flow may be achieved, Van Wazer, Lyons, Kim & Colwell (1963).

Consequently, the experimental procedure is to carry out aggregation in a Couette viscometer, counting the total number of particles at frequent intervals.

In a Couette viscometer in which the streamlines are circular the velocity gradient radially causes particles to be brought together. This differential velocity gives rise to a force which tends to push the particles together until the faster moving particle passes the radius on which the centre of the other particle lies. Thereafter, this force changes sign and tends to reseparate the particles. Allan & Mason (1962) have shown that this process will never of itself lead to the adhesion of two particles because the approach and recession paths of the particles are geometrically and kinetically symmetrical. If, however, an attractive force exists between the particles, collision may lead to the formation of an adhesion, because the interaction is no longer symmetrical. The conditions for this are that (i) during the phase of approach, the particles
come close enough for the adhesive forces to act, and (ii) as a result of (i), the reseparating force is never sufficient to overcome the newly established adhesion. It should be appreciated that the adhesive forces may be drawing the particles closer together during the phase of action of reseparating forces. Since the reseparating forces increase as \( \sin \theta \) with the angle \( \theta \) between the centre of the particles and the radial direction, the reseparating forces may at some value of \( \theta \) increase beyond the adhesive forces between the particles.

A. S. G. Curtis & L. Hocking (in preparation) have analysed the hydrodynamic forces acting on a pair of particles during collision (the treatment is similar to that given by Allan & Mason, 1962) and the effect which a given force of attraction has on the symmetry of such a collision. If the force of attraction follows an inverse square relationship with distance and is the London-Hamaker force, Curtis & Hocking find that a force constant is given by the relationship

\[
M = 72\pi \mu a^2 H,
\]

where \( H \) is a function of the stability ratio, given by \( H = 10^{1-178} \sqrt{\nu} - 10^{-86} \) (for the retarded force), \( \mu \) the viscosity of the suspending medium, \( a \) the radius of the interacting particles and \( G \) the shear rate. Similar expressions could be derived for other forces of attraction.

Thus in general the energy of adhesion can be written

\[
V_A = -f \frac{(M)}{d^2},
\]

(the energy of adhesion is conventionally represented as of negative sign) assuming that any attractive force is proportional to \( 1/d^2 \) where \( d \) is the half distance between the particles at their closest approach at equilibrium. If the force of adhesion be identified with the London force, \( V_A \) may be written (using the parallel plate solution)

\[
V_A = \frac{-M}{48\pi d^2} = \frac{-1.5\mu a^2 GH}{d^2} \text{(erg/cm}^2\text{)}
\]

This treatment may be used whether adhesion takes place with molecular contact between surfaces, or in the secondary minimum, provided in the latter case that a scale factor is introduced to allow for the fact that the experimentally determined value of \( M \) refers, not to the true surface, but to the outer edge of the double layer. However, if adhesion is in the primary minimum (molecular contact) and a marked potential energy barrier to close approach of the surfaces is found, this treatment cannot be used. In such a case the collision efficiency is a measure of the height of the potential energy barrier and a treatment based on Fuchs's theory would be more appropriate. But it is probable that in many cases, in particular in biological situations, adhesion is not of this type (Curtis, 1966) but rather adhesion with the particles in molecular contact and an inappreciable potential energy barrier, or adhesion in the secondary minimum.
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If adhesion is in the primary minimum $M$ can be identified with the London Hamaker constant. If no assumptions are made about the mechanism of adhesion the value of $H$ gives a quantitative value which may be used in the comparison of adhesiveness.

Equations (4) and (6) indicate that the collision efficiency for a given adhesiveness decreases logarithmically with increase of shear rate or the cube of the particle radius. Thus when the adhesiveness of two cell types are being compared either differences in $a$ and $G$ must be taken into account or measurements must be carried out with identical values of $G$ and $a$. These relations between the particle radius and collision efficiency indicate that if two cell types are of equal adhesiveness the larger cell type will be found to give the smaller stability ratio (collision efficiency). It is of interest to note that if the collision probability is a measure of the probability of a potential energy barrier being surmounted (Fuchs's theory) then there will be no dependence of the stability ratio on shear rate because the rate determining step, which is dependent on the frequency with which the Brownian motion energy exceeds a certain value, is not dependent on shear rate. In order to obtain the adhesiveness for the adhesion between two individual cells the adhesive energy per cm$^2$ is divided by the interaction area.

As collisions take place aggregates will build up if the stability ratio has a value $\neq 0$. Eventually aggregation might be expected to lead to the accumulation of all the particles into a single aggregate. As this point is approached the kinetics of aggregation cease to obey equation (3). But in practice another process usually will intervene at an earlier stage of aggregation to cause the kinetics to diverge from this relationship. Albers & Overbeek (1960) discussed the theory of the effect of shear forces in breaking up aggregates. They concluded that the shear forces acting on an aggregate body due to the difference in velocity of flow of the medium on either side of the aggregate will increase as the square of the radius of the aggregate. Consequently for a given adhesiveness or value of the stability ratio and a given value of the shear rate $G$ there will be an equilibrium size of aggregate at which the rate of break up equals the rate of aggregation. As aggregates form there will be a marked divergence of the kinetics from those indicated by equation (3) as the equilibrium condition is approached. When aggregation has reached equilibrium conditions particles of all sizes from single membered ones through aggregates up to the limiting size will be present. But the majority of particles will be present in the largest aggregates. Thus the greater the value of $G$ the smaller will be the maximum size of the aggregates at equilibrium although the aggregation rate given by equation (3) will be greater. Thus if two aliquots of the same cell suspension are reaggregated at different shear rates, the sample aggregated at a higher value of $G$ will aggregate more quickly and thus be judged more adhesive on this criterion whereas the equilibrium size of its aggregates will be smaller, and on this second criterion it will be judged the less adhesive. Earlier workers have
made subjective assessments of the adhesiveness of cells from the diameter of aggregates formed in a shaker after a certain period (Moscona, 1961) or from the initial rate of incorporation of single cells into aggregates using a shaker (Curtis & Greaves, 1965): it is clear now that not only can there be no comparison between the two techniques unless measurements be made either at the same value of \( G \) or unless differences in the value of \( G \) be taken into account, but that measurements using the shaker technique are of little value unless the value of \( G \) be constant throughout a series of experiments. Since variations in the viscosity of the medium will produce varying values of \( G \) for suspensions shaken at the same rate it is clear that many earlier conclusions may be erroneous. Examinations of equations (2) and (3) suggest that if a plot of \( \ln N_{\infty t} \) as ordinate and \( t \) as abscissa is made a linear relation should be obtained, provided that aggregation has not begun to approach equilibrium conditions.

In the discussion of the theory of this technique of measuring adhesiveness it has so far been assumed that shear conditions are alone responsible for the production of collisions between particles. However, in all suspensions Brownian motion tends to produce collisions between the particles. The collision rate is given by the relationship

\[
b_{ij} = \frac{2 kT}{3 \eta} \left( \frac{1}{r_i} + \frac{1}{r_j} \right) n_i n_j. \tag{7}
\]

Tuorila (1927) has shown that the ratio \( J/I \) of collisions due to shear to those due to Brownian motion is given by the relationship

\[
\frac{J}{I} = \frac{\eta (r_i + r_j) 3 G}{2 kT} \tag{8}
\]

where \( \eta \) is the viscosity of the medium and \( kT \) has the usual meaning. Thus for particles of radius 5 \( \mu \) with a shear rate \( G = 1 \) sec\(^{-1}\) and \( T = 298 \) °K less than 2 \% of the collisions are due to Brownian motion.

Thus if the kinetics of aggregation are examined under conditions where Brownian motion is unimportant in producing collisions and where aggregation has not begun to approach the equilibrium condition they should be described by equation (3). Therefore a plot of \( \ln N_{\infty t} \) should give a straight line, and the plot of collision efficiency for a given cell type as \( \ln \alpha \) against \( G \) should also give a straight line if the theory outlined above is correct.

For these reasons the technique for measurement of the stability ratio for the adhesion of cells is to measure the total particle concentration at frequent intervals before the onset of equilibrium conditions, under known values of shear in a Couette viscometer.

\[(b) \text{ The Couette viscometer}\]

This instrument consists essentially of a pair of concentric cylinders of radial dimensions such that when the smaller is suspended freely inside the larger a narrow gap exists between the two cylinders. The suspension of particles
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whose adhesiveness is to be measured is placed in this gap between the concentric cylinders and one of the cylinders is rotated. If the dimensions of the cylinders are chosen according to the principles described by van Wazer et al. (1963) laminar shear flow will be established in the gap when one cylinder is rotated relative to the other. The shear rate $G$ is determined by the rate of rotation of the cylinder and the radial dimensions of the cylinders.

A Couette viscometer was constructed by Mr. E. German of the Department of Engineering, University College London. The concentric cylinders are constructed of S90 stainless steel, which is resistant to corrosion by saline solutions, and the inner surface of the outer cylinder and the outer surface of the inner cylinder are machined to a surface finish of 10 $\mu$. The outer cylinder is rotated by means of an electric motor and a gearbox placed below it. In order to ensure a constant rate of shear an integrating motor (Ether Ltd., Stevenage, Herts.) which runs at a very stable speed, is used to drive the cylinder. The final rate of rotation of the outer cylinder can be varied between 1 rev/sec and 1 rev/min by appropriate choice of the voltage the motor is run at, or of the gearbox ratio: these low rates of rotation give shear rates between 1·4 sec$^{-1}$ and 84 sec$^{-1}$ with the radii of the inner and outer cylinders being 19·0 and 20·5 mm respectively. These low rates of shear allow the production of moderately large aggregates with various types of cell and permit a fairly long series of measurements to be obtained before equilibrium conditions are approached. The instrument is shown in Fig. 1 A.

In use the viscometer is placed in a constant temperature room some hours before measurements are to be undertaken. The inner cylinder or bob is suspended freely inside the outer cylinder on a torsion wire and before use of the instrument the inner bob is carefully centred inside the outer cylinder. The outer cylinder is carefully levelled so that its axis is aligned vertically. If the cylinders are misaligned the bob will rotate backwards and forwards (swing) through a certain angular excursion and variable shear conditions will be obtained.

Some of the later measurements have been made with other Couette viscometers made to a similar design by Associated Metalwork Ltd., Glasgow; these instruments are essentially identical with that described above. It might be possible that the stainless steel of the cylinders would yield heavy metal ions that would damage or kill the cells whose adhesiveness was being measured. It was, however, found that the activity of the enzyme ficin which is very sensitive to the presence of heavy metal ions was unaffected by incubation in the viscometer.

(c) Biological methods

Cell suspensions were obtained from two main sources: (i) the organs of embryonic chickens or (ii) from 'monolayer' tissue cultures. The method of preparation of viable single cells from chick embryonic tissue was as follows.
Fig. 1(A). General view of the Couette viscometer. A, the outer cylinder, B, the inner cylinder or bob of the viscometer. (B) Freshly dispersed 7-day embryonic chick liver cells under dark ground illumination on a Fuchs–Rosenthal haemocytometer (scale is given by the squares whose sides are 250 μ long). (C) Freshly dispersed 7-day embryonic chick neural retina cells under phase contrast illumination on a Fuchs–Rosenthal haemocytometer. These two photographs illustrate the high proportion of single cells obtained in these dispersions.
After dissection of the tissues in Hanks's saline, the organs to be dispersed were cooled to 2-4 °C. and washed three times with a modified calcium and magnesium free Hanks's saline (CMF). (Sodium chloride 0.119 M, Potassium chloride 0.004 M, Glucose 0.0055 M, Disodium hydrogen phosphate 0.0008 M, Potassium dihydrogen phosphate 0.0017 M, 2-amino (2 hydroxymethyl) 1,3 propanediol 0.025 M, pH 8.0.) They were then transferred to a 0.001 M di-aminoethane tetracetate solution in modified CMF (made isotonic by adding 14 % distilled water to the CMF) at 2 °C. The tissues were exposed to this solution for various times (e.g. liver 2 min, limb-buds 7 min, heart 10 min) and then the EDTA solution was replaced with the modified CMF saline. During this and all subsequent stages until reaggregation the cells were kept at 2-4 °C. After two further washes with this saline the tissues were dispersed mechanically by drawing them into and expelling them gently from a pasteur type pipette some twenty times. The success of this technique depends upon (a) starting with a pipette of orifice ca. 2 mm diameter, and using in turn pipettes of ca. 1 mm and 0.5 mm diameter orifice as the tissue breaks up and (b) ensuring that no air bubbles are formed in the cell suspension. The resulting suspension contains small aggregates, single cells, erythrocytes, a certain amount of debris from broken cells and intercellular material. Cell suspensions composed substantially of single cells were prepared by first sedimenting the aggregates from the suspension by centrifugation at ca. 20 g for 1 min: this technique also removes the majority of the erythrocytes. The supernatant from this centrifugation was taken and further diluted with CMF saline and then centrifuged at 100 g for 5 min. The supernatant which contains cellular debris etc. and some single cells was removed and discarded and the pellet resuspended in CMF saline to give the final cell suspension. In this way the cellular debris was reduced to a very low concentration. About 85 % of the population was recovered as single cells and the final suspensions contained > 95 %, usually more than 98 % single cells, see Fig. 1B, C. In later experiments the suspension was finally passed through an electroformed sieve (EMI Electronics Ltd., Hayes, U.K.) to remove aggregates, and these cell suspensions contained < 2 % of aggregates (expressed as proportion of cells in aggregates to total cell concentration). Cell suspensions were prepared from confluent or nearly confluent cultures of cell lines in tissue culture by a very similar technique. A calcium and magnesium-free saline and EDTA solution were prepared from the CMF medium. After exposure of the cultures to EDTA solution for 7 min this medium was replaced by the CMF medium. The cells were then dispersed by pipetting. Thereafter the cells were treated in the same manner as the embryonic tissue.

Cell viability was assessed by several criteria. In early work the respiration of the cells was measured (see Curtis, 1963). Electron micrographs were prepared of cell suspensions after they had been centrifuged into pellets to assist specimen preparation, see Fig. 2A, B. (These micrographs were taken by
Dr K. Vickerman.) The appearance of the cells in the electron micrographs suggests that they were alive at the time of fixation. The most extensively used technique was to carry out plating out tests of viability. The cells were plated out at very low population densities ca. $1 \times 10^3$/ml in a medium composed of 50% 199 medium, 10% horse serum (Burroughs Wellcome No. 2 Protein

Fig. 2(A, B). Two electron micrographs of freshly dispersed 5-day embryonic chick limb-bud cells. Fixed in glutaraldehyde-OsO₄ and post-treated with uranyl acetate and lead citrate. These electron micrographs were prepared by Dr K. Vickerman. (C). Section of an aggregate of neural retina cells from a 7-day chick embryo prepared in a Couette viscometer. Fixed 17 h after start of aggregation in Bouin's fluid. Stained with Harris haematoxylin. The relatively small size of the aggregate is due to the fact the aggregation was carried out at a high shear rate. (D). View of a cell suspension in a Couette viscometer, aggregation is fairly extensive. The rotation of the viscometer was stopped to obtain this photograph.
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concentration ca. 6.5%), 10% embryo extract and 30% Hanks's saline. After 1 h the proportion of cells which had settled and spread on the Petri dish base was measured. Cells which had settled and taken up a normal morphological appearance were judged to be alive. This technique probably gives an underestimate of viable cells because some cells which are alive probably do not settle and spread within 1 h. But if measurement of the proportion of spread cells is delayed for a longer interval after setting up the culture a falsely high value may be obtained owing to the contribution of mitosis to the viable cell population. Plating out tests showed that at least 63% of the cells were alive and in some experiments as many as 87% were alive. Finally histological preparations were made of some of the aggregates formed in these experiments: the cells in these aggregates appear to have been alive up to the time of fixation, see Fig. 2C.

The freshly prepared cell suspensions were diluted with three volumes of a saline composed of 50% Hanks's saline, 50% Medium 199, and then placed in the Couette viscometer. Reaggregation was carried out at 37 °C or at 1 °C or 2 °C. The cell suspensions were prepared so that the final population density of single cells was about 1·0–1·5 x 10⁶/ml. The population density of single cells and of all classes of particle (i.e. single cells, two celled and larger aggregates) was measured from a sample taken just before shear was applied to the cell suspension and at frequent intervals thereafter. Experiments with a given cell type were repeated a minimum of three times for any given conditions.

The measurements of the number of cells and aggregates per unit volume of the suspension were carried out using haemocytometers. Periodic checks were carried out to ascertain whether the total number of cells per unit volume decreased during aggregation. If evidence of such a loss of cells were found it might be due to lysis of cells, or adhesion to the walls of the viscometer or to settling of cells to the bottom of the viscometer, and it would lead to inaccuracies in the measurement of cellular adhesion. No evidence for such a loss was found save with BHK 21/C 13 and L 929 strain cells, which show appreciable settling within 15 min. Thus although results are included for the measurements of adhesiveness of these cells they may be rather inaccurate.

RESULTS

In all measurements of cell adhesion under the conditions used in this work the application of shear forces to suspensions of single cells led to their aggregation, see Fig. 3 and Fig. 2D. The time course of these aggregations when plotted with ln Nₐ as ordinate and t as abscissa, indicate that aggregation follows first order kinetics: in other words this plot gives a straight line relationship. This relationship is expected if aggregation kinetics are described by equation (3). Similarly when the stability ratios for a single cell type aggregated under a variety of different shear rates and otherwise constant conditions, are
Fig. 3. Plot of population density of cells and aggregates as $\ln N_\infty$ as ordinate against time from start of aggregation $t$ (min) as abscissa. The curve is a plot of the linear regression equation for the points, $\ln N_\infty = -0.026t + 2.11$, $s_b < 1 \times 10^{-4}$; cell type: neural retina. For convenience of plotting values of $N_m$ have been reduced by a factor of $10^5$.

Table 1. *Collision efficiency (stability ratio) for various cell types*

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Reaggregation medium</th>
<th>Temperature $^\circ\mathrm{C}$</th>
<th>Shear rate (sec$^{-1}$)</th>
<th>Mean collision efficiency (%)</th>
<th>$\sigma$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic chick tissues</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day ventricle</td>
<td>Hanks's + 199</td>
<td>37</td>
<td>3.60</td>
<td>3.8</td>
<td>0.5</td>
<td>9</td>
</tr>
<tr>
<td>5-day liver</td>
<td>Hanks's + 199</td>
<td>37</td>
<td>2.25</td>
<td>3.7</td>
<td>0.6</td>
<td>9</td>
</tr>
<tr>
<td>5-day limb-bud</td>
<td>Hanks's + 199</td>
<td>2</td>
<td>2.60</td>
<td>2.6</td>
<td>0.2</td>
<td>6</td>
</tr>
<tr>
<td>5-day limb-bud</td>
<td>Hanks's + 199</td>
<td>37</td>
<td>2.08</td>
<td>1.7</td>
<td>0.4</td>
<td>6</td>
</tr>
<tr>
<td>7-day liver</td>
<td>Hanks's + 199</td>
<td>37</td>
<td>11.46</td>
<td>9.8</td>
<td>1.9</td>
<td>5</td>
</tr>
<tr>
<td>7-day neural retina</td>
<td>Hanks's + 199</td>
<td>37</td>
<td>2.32</td>
<td>21.6</td>
<td>2.9</td>
<td>4</td>
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<tr>
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<td>5</td>
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<tr>
<td>7-day neural retina</td>
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<td>37</td>
<td>17.12</td>
<td>12.4</td>
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<td>Tissue culture lines</td>
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<td></td>
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</tr>
<tr>
<td>BHK 21/C13</td>
<td>Hanks's</td>
<td>37</td>
<td>5.18</td>
<td>14.5</td>
<td>1.9</td>
<td>4</td>
</tr>
<tr>
<td>L 929</td>
<td>Hanks's</td>
<td>37</td>
<td>5.18</td>
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</table>


plotted as In $\alpha$ against the shear rate a straight line relationship is obtained, see Fig. 4. These plots provide experimental evidence in favour of the theoretical basis of this technique of measuring adhesiveness. The finding of these relationships strongly suggests that aggregation kinetics follow the theory outlined earlier.

![Plot of collision efficiency $\alpha$ as a function of shear rate $G$. Ordinate, log $\alpha$, abscissa $G(\text{sec}^{-1})$. The curve is a plot of the linear regression equation for the points, log $\alpha = -0.0189G + 1.404$. Standard deviation of the regression coefficient $< 1 \times 10^{-6}$. Cell type: neural retina 7-day.](image)

Fig. 4. Plot of collision efficiency $\alpha$ as a function of shear rate $G$. Ordinate, log $\alpha$, abscissa $G(\text{sec}^{-1})$. The curve is a plot of the linear regression equation for the points, log $\alpha = -0.0189G + 1.404$. Standard deviation of the regression coefficient $< 1 \times 10^{-6}$. Cell type: neural retina 7-day.

Measurements of the stability ratio for a variety of cell types under a variety of conditions are given in Table 1. Plots of the stability ratio against time from the start of aggregation are linear and of zero gradient at the 0.05 probability level, at least until the total particle concentration has fallen below 25% of its initial value. At later stages of aggregation the measured stability ratio falls. Three reasons for this can be advanced, first that aggregation is approaching equilibrium conditions, secondly that the majority of cells have accumulated into a few aggregates and thirdly that the adhesiveness of cells changes after incubation for a certain period. Plots which illustrate this behaviour are shown in Fig. 5.
DISCUSSION

The technique for measurement of cellular adhesiveness described in this paper has considerable theoretical basis in the work of Swift & Friedlander (1964), Albers & Overbeek (1960) and Mason & Bartok (1959) on the interaction of particles at collision. The findings in this paper provide additional experimental support for the application of this technique to the measurement of cellular adhesiveness. It should be realized that the only assumptions that are made about the cells in this treatment are (i) that they are spheroidal, (ii) that

![Graph](image)

Fig. 5. Plot of population density of cells and aggregates in an aggregating system as $N_\infty$ against time from start of aggregation $t$ (min). $\ln N_\infty$ as ordinate, $t$ as abscissa. Cell type BHK 21 C13 cells. The fairly marked deviation of the plot from linearity is probably due to sedimentation of the cells towards the bottom of the viscometer from which region samples were removed. For convenience of plotting values of $N_\infty$ have been reduced by a factor of $10^5$. 
they are unable to move by themselves and (iii) that they have a density inappreciably different from the suspending medium. No assumptions are made about the mechanism of cell adhesion other than that there is no appreciable potential energy barrier to close approach in the cases where close (molecular contact) approach is found. It is possible in some systems that occasional events such as the action of Brownian motion collisions between the medium and the particle (Fuchs, 1934) or between the medium and protrusions from the particle (Pethica, 1961) may provide a mechanism whereby the potential energy barrier is surmounted. In such cases the effectiveness of collisions in producing adhesion will be a measure of the frequency with which the barrier is surmounted and will not be affected by the shear rate used to bring the particles into collision.

The finding that plots of $\ln N_{\infty}$ are linear (which is borne out by the small values of the standard deviation for values of the stability ratio of cell types not included in Fig. 3) indicates that aggregation of these cells obeys the kinetics predicted by equations (1) and (3). Similarly the relationship between the shear rate and the measured collision efficiency affords further evidence for the agreement of theory and experiment and in addition provides evidence that in these systems adhesion does not occur by the occasional surmounting of a large potential energy barrier to give adhesions with the two surfaces in the primary minimum (molecular contact). Furthermore, these results indicate that under the conditions of cell preparation and aggregation used in this work the adhesiveness of the cells does not change appreciably in the period over which the measurements were made. This technique could, however, provide a means of following rapid changes in cellular adhesion.

The theoretical interpretation of measurements of the collision efficiency was outlined earlier in this paper. It was shown that these measurements yield values of force constants which can be used to compare the adhesiveness of cells. In that section the force constant $M$ was evaluated on the assumption that the London-Hamaker force is the only force of attraction between the cells. Since the mathematical relationship of the value for the collision efficiency to the force constant is determined by the nature of the forces of attraction, it is essential to be able to recognize these forces in order to calculate the energy of adhesion. It is also necessary to know the distance of separation of the surfaces of the cells when the adhesion reaches a steady state. Although there is considerable circumstantial evidence that the force of attraction acting in cell adhesion is the London-Hamaker force (see Curtis, 1967), it is desirable to obtain independent evidence on this point. Examination of the theoretical basis of the method described in this paper suggests two methods of obtaining such evidence. The first method depends upon the relationship between the shear rate $G$ and the collision efficiency (see Fig. 4). The relationship indicates that the force of attraction declines as the inverse square of the distance between the surfaces. Of the various possible forces of adhesion only the London-
Hamaker forces shows this relationship. The second method depends upon
the internal consistency of the values of the London-Hamaker constant cal-
calculated from the measured values of the collision efficiency with the range of
values of the constant known from other methods of measurement and
theoretical calculation. Overbeek (1952) has shown that the constant has
values in the range $1 \times 10^{-15} - 10^{-12}$ erg. Thus if the value of $M$ derived from the
measured collision efficiency falls in this range there is further reason for
concluding that the London-Hamaker force acts in the adhesion being studied.
There are, however, two reasons why the value of $M$ may fall outside this
range. The first of these is that the force of attraction is not the London-Hamaker
force. The second is that adhesion takes place in the secondary minimum be-
cause a potential energy barrier due to an electrical double layer prevents close
approach of the surfaces. If adhesion takes place in the secondary minimum
a low value will be found for the apparent London-Hamaker constant. Such
an apparent constant can be converted to the true constant if the distance of
separation of the cells is known.

Table 2. Energies of adhesion for various cell types

<table>
<thead>
<tr>
<th>Cell type</th>
<th>$H$ (erg/cm$^2$)</th>
<th>$G$ sec$^{-1}$</th>
<th>London-Hamaker constant $M$ (10$^{-17}$ erg)</th>
<th>Adhesive energy (erg/cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryonic chick tissues</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-day ventricle</td>
<td>$7.35 \times 10^{-9}$</td>
<td>3.60</td>
<td>$2.21 \times 10^{-17}$*</td>
<td>$1.47 \times 10^{-5}$†</td>
</tr>
<tr>
<td>5-day liver</td>
<td>$7.08 \times 10^{-9}$</td>
<td>2.25</td>
<td>$4.06 \times 10^{-17}$*</td>
<td>$2.61 \times 10^{-5}$†</td>
</tr>
<tr>
<td>5-day limb-bud at 2 °C</td>
<td>$1.90 \times 10^{-9}$</td>
<td>2.60</td>
<td>$8.41 \times 10^{-18}$*</td>
<td>$5.66 \times 10^{-6}$†</td>
</tr>
<tr>
<td>5-day limb-bud at 37 °C</td>
<td>$8.89 \times 10^{-10}$</td>
<td>2.08</td>
<td>$3.14 \times 10^{-18}$*</td>
<td>$2.93 \times 10^{-6}$†</td>
</tr>
<tr>
<td>7-day liver</td>
<td>$9.51 \times 10^{-8}$</td>
<td>11.46</td>
<td>$1.76 \times 10^{-18}$*</td>
<td>$1.17 \times 10^{-4}$†</td>
</tr>
<tr>
<td>7-day neural retina</td>
<td>$7.58 \times 10^{-6}$</td>
<td>2.32</td>
<td>$1.97 \times 10^{-15}$</td>
<td>$2.09 \times 10^{-2}$</td>
</tr>
<tr>
<td></td>
<td>$1.29 \times 10^{-4}$</td>
<td>4.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$9.89 \times 10^{-7}$</td>
<td>7.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$8.81 \times 10^{-7}$</td>
<td>12.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$5.87 \times 10^{-7}$</td>
<td>17.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tissue culture lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHK 21/C 13 at 37 °C</td>
<td>$8.64 \times 10^{-7}$</td>
<td>5.18</td>
<td>$2.50 \times 10^{-15}$</td>
<td>$2.62 \times 10^{-2}$</td>
</tr>
<tr>
<td>BHK 21/C 13 at 2 °C</td>
<td>$5.17 \times 10^{-4}$</td>
<td>5.18</td>
<td>$1.50 \times 10^{-14}$</td>
<td>$1.59 \times 10^{-1}$</td>
</tr>
<tr>
<td>L 929</td>
<td>$8.51 \times 10^{-4}$</td>
<td>8.20</td>
<td>$2.47 \times 10^{-14}$</td>
<td>$2.60 \times 10^{-1}$</td>
</tr>
</tbody>
</table>

* These values are probably not of the true London-Hamaker constant, but represent
a pseudo-constant, see discussion.
† Calculated on assumption that adhesion is in the secondary minimum at $2d = 100 \text{Å}$.

Adhesive energies, force constants $M$ and values of $H$ have been calculated
from the measured values of the collision efficiencies on the assumption that
the force of adhesion is the London-Hamaker force. These values are given
in Table 2. The values of the force constant (London-Hamaker constant) for
7-day embryonic chick neural retina cells, BHK 21/C13 and L 929 cells are
consistent with probable values for this constant. This consistency also suggests
that adhesion occurs with these cells in molecular contact under the conditions of the experiment. Energies of adhesion have been calculated from these values of the constant on the assumption that the particles adhere at a separation of 5 Å. The values for the apparent London-Hamaker constant for the other cell types given in Table 2 are surprisingly low. It is tentatively suggested that these values indicate that the cells are adhering in the secondary minimum. Energies of adhesion for these cells have been calculated on the assumption that adhesion is in the secondary minimum with the separation of the cell surfaces equal to 100 Å. These energies are of the order \(10^{-6}-10^{-4}\) erg/cm² that would be expected if adhesion occurs in the secondary minimum with the true London-Hamaker constant of values in the range \(10^{-15}-10^{-14}\) erg, a surface potential in the range \(-10\) to \(-20\) mV and ionic conditions approximating to those found in the Hanks' and 199 medium (see Curtis, 1967). It should be appreciated, however, that these values for the energy of adhesion in the secondary minimum are considerably less exact than those for the neural retina, BHK 21/C 13 and L 929 cells, because the value of the distance of separation of the cells is not known. In calculation of these adhesive energies it is possible to ignore electrostatic repulsive forces with only a small increase in error. The reasons for this are (i) when adhesion occurs in the primary minimum electrostatic forces of repulsion will be relatively small for a particle of the diameter of a cell (Curtis, 1967), (ii) when adhesion occurs in the secondary minimum there is a negligible electrostatic force of repulsion preventing approach of the surfaces as far as their adhesion. Since the electrostatic force falls off exponentially the only applicable force leading to the approach of two surfaces from a very large separation to the ‘bottom’ of the secondary minimum will be the London-Hamaker force. The error introduced by neglect of the electrostatic force will be relatively small. The calculation of the adhesive energy for those cells which appear to adhere in the secondary minimum was performed by treating the apparent London-Hamaker constants as the true ones and by applying a scale factor for the separation of the cells to correct for the fact that the measured constant refers not to the true surface but to the edge of the double layer.

The energies of adhesion are expressed in erg/cm² of contact surface. Since the actual contact areas of the cells are not known it is most useful to express the energies of adhesion in this manner. In the theoretical treatment by Curtis & Hocking the particles are treated as spheroids in their interactions which may or may not lead to the establishment of adhesions. Thus the contact area involved in the establishment of an adhesion may be defined geometrically from the radius of the particles, and the force of attraction based on this area. Once an adhesion has formed the cells depart from the spherical shape and the contact area presumably extends. Thus the measurement of adhesiveness which is obtained by this technique is not only an average value for a large cell population, but represents an average value for the surface of a single cell,
should the situation arise in which the adhesiveness varies from one part of a cell to another. Unless the contact area is known the total energy of adhesion of a cell cannot be calculated. Assuming a cell to cell contact area of 300 μ² (Curtis, 1967) the weakest adhesions measured have a value of the order of 10⁻¹¹ erg/cell contact (i.e. circa 100 kT). It is of interest that Brooks et al. (1967) found values for the force of adhesion between tissue cells using a dispersion technique, which are not inconsistent with the adhesive energies measured in the present work for those cells which apparently adhere in the secondary minimum. Recently Weiss (1968) has extended the technique used by Brooks et al. (1967) and has found evidence that cell to glass adhesions may be either in the secondary or primary minimum.

It should also be realized that even when there is no clear evidence as to the nature of the adhesive force this technique for measuring cell adhesiveness provides a measurement of a force constant as a function of the collision efficiency. This can be used for comparing the adhesiveness of two cell types quantitatively provided that cells are of the same radius and that the measurements are made at the same shear rate. If measurements are made at different shear rates or for cells of different size these factors may be taken into account by the use of equation (4), or similar expressions if other forces of adhesion act. It appears that it has not previously been realized that in order to compare the adhesiveness of two cell types it is necessary to take these factors into account. A further advantage of this technique is that it allows the detection of rapid changes in cell adhesiveness, although in the present work no evidence was found of changes in cell adhesiveness under the conditions of preparation of the cell suspensions, of culture of the cells or of their measurement.

These measurements of the stability ratio (collision efficiency) for the adhesion of cells show that it may be incorrect to assume as did Wilkins, Ottewill & Bangham (1962) that every collision is effective in producing an adhesion, and provide data to resolve the difficulty that Steinberg & Roth (1964) experienced in their treatment of flocculation kinetics of cells in being unable to put any value to the collision efficiency.

**SUMMARY**

1. A new method for the quantitative measurement of cellular adhesiveness is described. This technique provides a measurement of the probability that a collision between two cells or groups of cells will result in their adhesion. This measurement of the collision efficiency (stability ratio) yields an average measurement of the adhesiveness of a population of cells. The relationship between the collision efficiency and the energy of adhesion of the cells is described.

2. The technique depends upon the comparison of the actual rate of formation of aggregates with the rate at which collisions take place. This latter rate can be obtained if the cells are aggregated under conditions of constant shear rate
Measurement of cell adhesiveness

of known value. Aggregation is carried out in a Couette viscometer which provides conditions of constant shear.

3. The analysis of the measurements of collision efficiency is discussed and it is shown that the method of measurement can be applied to measure the energy of adhesion for a variety of types of adhesion.

4. The theory of the analysis of the experimental measurements shows that values of the collision efficiency (and other methods of measuring adhesiveness) for a given energy of adhesion are dependent on the shear rate under which measurements are made, and on the diameters of the cells. A technique is given for the comparison of measurements of the collision efficiencies obtained for cells of different sizes or under differing shear rates.

5. The relationship between shear rate and the measured collision efficiency suggests that the London-Hamaker force acts in the adhesion of cells. The energies of adhesion and London-Hamaker constants calculated from the collision efficiencies on the assumption that this force acts are given for a variety of cell types. Adhesive energies fall either in the range $10^{-6}-10^{-4}$ or $10^{-2}-10^{-1}$ erg/cm², corresponding probably to adhesion in the secondary and primary minimum respectively.

6. The technique can be used to obtain measurements of the adhesiveness when this is changing relatively rapidly. The technique of measurement produces aggregation of the cells under conditions essentially similar to those used in standard techniques for reaggregation.

RéSUMÉ

Le mesurage de l’adhésion cellulaire par une méthode absolue

1. On décrit une nouvelle méthode pour le mesurage quantitatif de l’adhésion cellulaire. Cette technique fournit une mesure de la probabilité qu’une collision entre deux cellules ou groupes de cellules mène à leur adhésion. Ce mesurage de l’efficacité de collision (la proportion de stabilité) produit une mesure moyenne de l’adhésion d’une population de cellules. Le rapport entre l’efficacité de collision et l’énergie de l’adhésion des cellules est décrit.

2. La technique dépend de la comparaison du taux véritable de formation d’agrégés avec le taux auquel les collisions se produisent. On peut obtenir ce dernier taux si les cellules s’agrègent sous des conditions de taux constant de cisaillement de valeur connue. L’agglomération s’effectue dans un viscomètre de Couette qui fournit des conditions de cisaillement constant.

3. On discute l’analyse des mesures de l’efficacité de collision et on montre que la méthode de mesurage peut s’appliquer à mesurer l’énergie de l’adhésion pour une variété de types d’adhésion.

4. La théorie de l’analyse des mesures expérimentales montre que les valeurs de l’efficacité de collision (et des autres méthodes pour mesurer l’adhésion) pour une énergie déterminée de l’adhésion dépendent du taux de cisaillement.
sous lequel on fait les mesures, et des diamètres des cellules. Une technique se présente pour la comparaison des mesures des efficacités de collision obtenues pour les cellules des dimensions différentes ou sous des taux de cisaillement divers.

5. Le rapport entre le taux de cisaillement et l'efficacité de collision mesurée suggère que la force London-Hamaker agit dans l'adhésion de cellules. Les énergies d'adhésion et les constantes London-Hamaker, calculées des efficacités de collision sur la supposition que cette force agit, sont fournies pour une variété de types de cellules. Les énergies adhésive se trouvent ou dans la gamme $10^{-6} - 10^{-4}$ ou $10^{-2} - 10^{-1}$ erg/cm², qui correspondent probablement avec l'adhésion dans le minimum secondaire et premier respectivement.

6. On peut utiliser la technique pour obtenir des mesures de l'adhésion quand celui-ci change relativement rapidement. La technique de mesure produit l'agglomération des cellules sous les conditions essentiellement pareilles à celles employées dans les techniques classiques pour le réagglomération.

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REFERENCES


Measurement of cell adhesiveness


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