THE MECHANISM OF ADHESION OF CELLS TO GLASS

A Study by Interference Reflection Microscopy

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ABSTRACT

An optical technique for measuring the thickness of thin films has been adapted and evaluated for studying the structure of the adhesion of cells to glass in tissue culture. This technique, which is termed interference reflection microscopy, has been used to study embryonic chick heart fibroblasts. These findings have been observed: in normal culture medium the closest approach of the cell surface to substrate in its adhesions is *ca.* 100 A, much of the cell surface lying farther away; chemical treatments which bring the cell surface to near its charge reversal point reduce the closest approach of adhesions to <50 A, probably to <30 A; chemical treatments which increase surface charge increase the nearest approach of cell and substrate in adhesions from *ca.* 100 A; high osmotic concentration of a non-polar substance, *i.e.* sucrose, does not affect the distance between cell and substrate in the adhesions. In addition, optical evidence indicates that there is no extracellular material between cell and glass in the adhesions. When cells de-adhere from glass, they appear not to leave fragments behind. The adhesive sites in these fibroblasts appear to be confined to the edge of the side of the cell facing the substrate and to the pseudopods. The significance of this is discussed in relation to the phenomenon of contact inhibition. Evidence is presented that the mechanism of cell adhesion does not involve calcium atoms binding cells to substrate by combining with carboxyl groups on cell surface, substrate, and with a cement substance. Osmium tetroxide fixation results in a final separation of 100 to 200 A between cell and substrate: there are reasons for thinking that this fairly close approach to the condition in life is produced as an artefact. The results can be accounted for only in terms of the action of electrostatic repulsive forces and an attractive force, probably the van der Waals—London forces. Biological arguments suggest that these results are equally applicable for cell-to-cell adhesions.

INTRODUCTION

The results of electron microscopy of tissues suggest that a gap 100 to 200 A wide is often found between the plasma membranes of two cells apparently in contact and adhesion. At present, views differ as to the nature of this gap. If the gap exists in life, its properties and the functions it serves are of great interest in relation to the question of cell adhesion. Many of the theories of adhesion so far advanced accept that the gap exists in life, and they are so expressed that they are able to account for its occurrence. However, certain experiments, *e.g.* those of Wilkins, Ottewill, and Bangham (1), can be interpreted to imply that the gap is a fixation artefact. If this is so, it is of

considerable interest to confirm their results by other means and to examine the mechanism by which this artefact arises. In consequence, the present work has been directed to discovering whether the gap is found in life, if so, what factors are involved in its maintenance, and thus what light can be thrown on the problem of cell adhesion.

Three main interpretations of the nature of the gap can be made. First, it can be assumed that the gap exists in life and is filled with some substance which binds cell to cell by chemical bonds. Robertson (2), reporting on the consequence of treating myelin with hypo- and hypertonic solutions, expressed the view that the gap is filled with a hydrated colloid which helps to bind the cells together. Several other theories of cell adhesion have supported this interpretation. As a second interpretation Pethica (3) suggested that in life the cells come into contact by their plasma membranes so that the gap found in electron micrographs either is an artefact or is due to a misinterpretation of plasma membrane structure. This concept is implied in the discussion of experiments on the flocculation of sheep polymorph leucocytes given by Wilkins, Ottewill, and Bangham (1). These authors found that if the surface charge of such cells was suppressed with heavy metal ions, then flocculation occurred. Measurements of the flocculation rate were interpreted to mean that the cells came into adhesion, due to the flocculation, with no gap between them. It was claimed that these cells were alive, and thus that adhesion between living cells occurred with no gap between the cell surfaces. If their interpretation is correct, the gap found in electron micrographs is an artefact. A third point of view is that which I have suggested (4, 5), namely, that a 100 to 200 A gap is actually found in life and that, though intercellular material may be present in this gap, it is not present to such a degree as to form the main means of cell adhesion. In this theory, adhesion is thought to result mainly from the interaction of the long-range van der Waals–London forces between the cell surfaces and the repulsive forces due to the surface charges of the cell, according to the Derjaguin, Landau, Verwey and Overbeek theory of lyophobic colloid stability (see references 4, 5). At 100 A or so beyond a cell surface, the van der Waals–London forces are larger than the repulsive forces; in consequence, two surfaces are drawn together until they are about 100 A apart. There are reasons for believing that the adhesion of cell to cell does not differ fundamentally from that of cell to glass (see 4, 5).

If the plasma membrane of a cell growing on glass possesses a refractive index which differs from that of the film of intercellular medium between cell and glass, the distance of separation between the cell and glass can be measured by optical methods. Hereafter, the thickness of this and "true" intercellular gaps will be referred to as the gap or interphase thickness. The theoretical aspects of the optical methods have recently been re-analysed by Vasicek (6). Van den Tempel (7) used such a method to measure the gap between two apposed oil globules; but such methods do not appear to have been used previously in biological research. This technique may be termed interference reflection microscopy. It is suitable for measuring separations down to *ca.* 50 A (with a clear indication of smaller thicknesses if they occur), and can thus be used to investigate the relations of cells adhering to a surface, and may elucidate the general mechanism of cell adhesion.

MATERIALS AND METHODS

In essence, an intense monochromatic beam of collimated light is arranged to strike a series of interfaces at normal incidence; the intensity of reflection in the axis of the incident light is related to the separation of the interfaces. The phase differences between the light reflected from one interface and another interact to produce an interference effect. In a system composed of three media of refractive index, n_0, n_1, and n, where two outer phases are of considerable depth but the intermediate one is of small thickness d and refractive index n_1, the intensity p of light reflected (relative to the incident intensity) normally after interference is, according to Vasicek (6), given by:

$$p = \frac{\left\{ n_1^2(n - n_0)^2 \cos^2\left(\frac{\pi}{\lambda} 2n_1 d \cos\theta\right) + (n_1^2 - n_0 n)^2 \sin^2\left(\frac{\pi}{\lambda} 2n_1 d \cos\theta\right)\right\}}{\left\{ n_1^2(n + n_0)^2 \cos^2\left(\frac{\pi}{\lambda} 2n_1 d \cos\theta\right) + (n_1^2 + n_0 n)^2 \sin^2\left(\frac{\pi}{\lambda} 2n_1 d \cos\theta\right)\right\}} \quad (1)$$

λ is the wavelength of light used, θ is angle of incidence, and $\theta = 0°$ for normal incidence (convention

used by Vasicek). If cells are grown on a glass surface, which can be taken as representing the medium of refractive index n_0, the gap (if present) will have refractive index n_1. The refractive index of the glass used is known (n_0 = 1.515), various assumptions can be made about that of the gap, which have interesting biological implications (see below), and values for the outer regions of the cell can be obtained from surface contact microscopy (8). Although protein may be absorbed to the glass, there is reason to think (9) that this film is less than 20 A thick, which will hardly affect measurements by this method.

The optical equipment used is shown in diagram in Fig. 1. A 1 kw high pressure mercury arc lamp run from a d-c supply with a large ballast resistance was used as a light source. An interference filter (Baltzers: Calflex) removed infrared radiation, an ultraviolet absorbing filter, and a 5461 A interference filter reduced the light output to a narrow band centered near 5461 A. This light was collimated with an $f/1.9\lambda$ lens onto a field iris, injected into the microscope body above the objective through an auxiliary lens, an aperture iris, and a half-silvered mirror centrable in the optic axis of the microscope. By this means an intense collimated beam fell on the back of the objective and illuminated the object as a convergent cone. Since the most oblique rays in the cone actually falling on the object did not diverge more than 12.5° from normal incidence because of the small diameter and accurate centering of the incident beam on the back of the objective, values of cos θ in Equation 1 do not differ appreciably from the values for normal incidence. The cone angle was determined by inserting in the illuminating beam a mask allowing ½ or ⅛ field illumination. The form and dimensions of the illuminating cone were then determined at the front surface of the objective photographically. The distance from coverglass to front surface was measured with a micrometer. Stopping down of the aperture was used to check that axiality was observed. From these measurements the cone angle could be calculated. In addition, the cone angle was measured by projecting the beam from the objective through an oiled-on glass block onto cards placed at various distances below the objective. In consequence, equation 1 can be applied to this measuring system (see also Vasicek, reference 6). By opening the iris the cone angle could be increased and the image destroyed by the interferences at

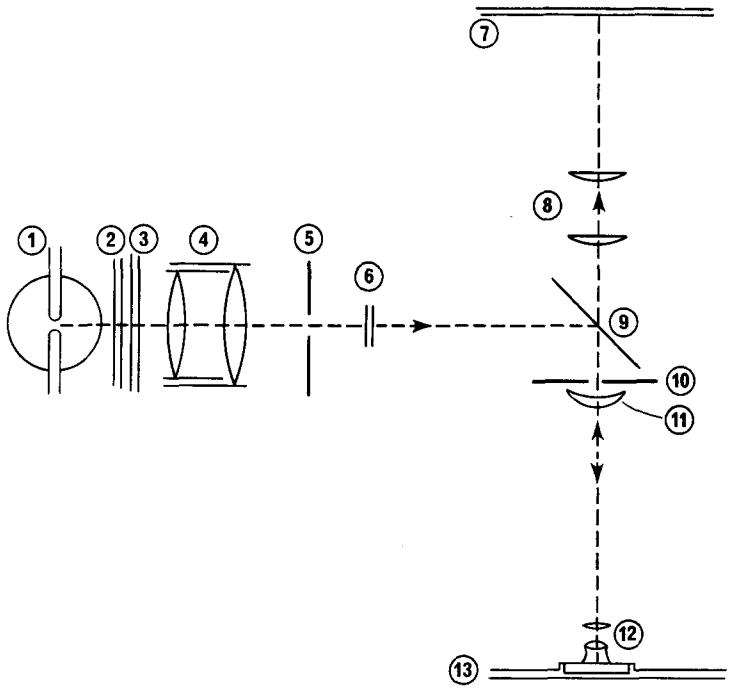


FIGURE 1 Diagram of optical equipment. 1, mercury arc lamp; 2, heat reflecting interference filter; 3, ultraviolet absorbing filter; 4, collimating lens; 5. field iris; 6, 5461 A line isolating filter; 7, photographic plate; 8, projection eyepiece; 9, half silvered mirror; 10, aperture iris; 11, auxiliary lens; 12, objective (immersion); 13, slide with hanging drop culture. Not drawn to scale.

many different angles of incidence. A 50 ×, na 1.0 oil-immersion fluorite objective was used.

The intensity of reflection was measured photographically. With a photomicrographic attachment, Kodak P 1600 plates were exposed for 15 seconds (this long exposure tends to cut down the percentage standard deviation in exposure length due to inaccurate timing over short intervals).

Development was carried out under standardised conditions: the plates were developed for 11 minutes in Kodak D 76 at 20°C with continuous agitation. An emulsion characteristic curve was prepared with each batch of plates (*i.e.* those exposed within 1 hour) by exposing a plate through a series of density steps. The density steps were in turn calibrated on a spectrophotometer (Hilger, Uvispek). The emulsion densities were measured after development on a Joyce-Loebl recording microdensitometer, and from their values characteristic curves could be prepared. Plates were exposed at a temperature of 36°C.

Normal embryonic chick heart fibroblasts from 9-day embryos were grown on glass in a medium composed of 2 parts cockerel serum, 1 part extract of 9-day embryos, 1 part Pannett-Compton saline. The cells were derived from primary explants. The glass culture surface was of borosilicate glass (Chance: resistance) which had been cleaned with boiling chromic acid (70 per cent H_2SO_4; 4 per cent CrO_3), followed by repeated boiling in distilled water, with final drying and sterilization under an ultraviolet lamp. This method of cleaning produces a very hydrophilic glass surface free from adsorbed chromic acid and routinely gives very healthy cultures.

When the cells had been grown for 24 hours they were examined by interference reflection microscopy. The cultures were grown as hanging drops in cavity slides, the bottom of the cavity being painted with optical matt black paint to minimize reflection from this surface. The cells were photographed with this method of microscopy and were then treated with one of the following reagents, after which treatment they were then photographed again to discover whether the reagents had altered the distance between cell and substrate.

1. 1.0 per cent osmium tetroxide buffered at pH 7.4 with Veronal buffer, (0.028 M sodium barbiturate 0.054 M sodium acetate).
2. 0.003 M cupric chloride made up in 0.050 M NaCl solution, unbuffered pH *ca.* 5.0.
3. 0.020 M sodium acetate–HCl buffer pH 3.6 in 0.050 M NaCl.
4. 3 M NaCl solution buffered at pH 7.2 with 0.002 M Tris-HCl.
5. 0.10 M calcium chloride made up in 0.050 M NaCl buffered at pH 7.0 with 0.002 M Tris-HCl.
6. 0.050 M NaCl buffered at pH 8.20 with 0.002 M Tris-HCl.
7. 0.001 M ethylenediaminetetraacetate (EDTA) buffered at pH 8.22 with 0.002 M Tris-HCl, in 0.05 M NaCl.
8. 0.5 per cent Difco trypsin dissolved in Hank's saline, Ca-Mg-free.
9. Distilled water.
10. 3 M sucrose solution buffered at pH 7.2 with 0.002 M Tris-HCl, in 0.050 NaCl.

The reasons for choosing these reagents appear in the Discussion. They were warmed to 36°C shortly before use, and the pH values refer to this temperature. Injection of these solutions was carried out by inserting a fine hypodermic needle through the wax seal of the cultures; at least 2 ml of any reagent was injected into a given culture in which the volume of culture medium was less than 0.1 ml.

Optical Calibration of the Equipment

Three tests were carried out on the optical system before it was used for measurements of light intensity. First, it was determined that no appreciable geometrical distortion was present in that central part of the image field used for measurements, by applying the technique described by Hallert (10). A test grid was prepared by Messrs. Graticules of London and tested by the National Physical Laboratory. In consequence, there no is reason to suppose that distortion might alter illumination levels in the various parts of the image field. Second, frequent tests for evenness of illumination across the field were carried out by photographing the field on a coverslip carrying a drop of water (focusing on the glass-water interface). Transects of these negatives were made with a microdensitometer and it was found that, except within 1 mm of the edge of the negative, image illumination had been even. Third, tests for the presence of glare and scattered light arising in the optical system and reaching the plate were performed by using the technique described by Curtis (11); it was found that stray light was never greater than density 0.005 (in the negative) above that which would be expected on the nature of diffraction within the object, and this density was never equal to more than a 2 per cent difference in illumination falling on the plate.

However, it is possible that diffraction effects arising within the object might cause a sufficient scatter of light to vitiate measurements. This problem cannot yet be solved theoretically because of the extreme complexity of the system, although Wilkins' treatment (12) for transmitted light and a condenser aperture na 0.5 suggests that it will not be serious. Here the na is 0.3 with reflected illumination. Nevertheless it would not be expected that diffraction effects would be serious in the central regions of a thin film. Diffraction effects might be more extensive at the edge of a film.

In Figs. 4 and 5, a series of parallel lines may be seen to follow the outline of the cell. Although these might be due to edge diffraction, they are more probably overhanging portions of the cell surface farther away from the glass (see Fig. 2). The main reason which supports this interpretation is that, using white light illumination, although the innermost band is black the bands peripheral to it are coloured, which indicates that they are higher orders due to the cell surface going farther away from the glass (an order = about 2000 A). In addition, these bands are invariably included in the outline of the cell as seen by transmitted light phase microscopy. Measurements of gap thickness have not been made on these bands which are too narrow for accurate densitometric measurement.

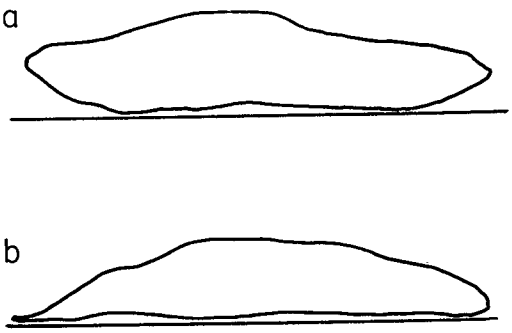


FIGURE 2 Diagram to illustrate probable origin of fringes at edge of cell. Parts *a* and *b* represent cells in section adhering to a glass surface. The cell in *a* would be expected to give rise to fringes because its undersurface steps away from the glass as an "overhang" at its edges. The cell in *b* would not be expected to give any fringes because the undersurface remains close (within one order) to the glass over its whole extent, and no parts of the undersurface are farther away than one order.

Optical Evaluation of the Results

Inspection of equation 1 makes it obvious that the value of the reflectivity depends on the values of n, n_1, and n_0, so that for a measured reflectivity the calculated gap thickness depends on these values. The first problem is raised by the correct selection of the values of these constants, and a second one by the difficulty of measuring the reflectivity, since it would be hard to measure directly with accuracy the intensity of incident illumination. Furthermore, a related problem is whether the cell surface and interior have sufficiently similar refractive indices for the three-component system represented by equation 1 to be adequate, in which n is taken as referring to both the cell surface and nearby interior.

The system was tested by placing a mica sheet (muscovite) with cleavage steps immersed in paraffin oil on a glass coverslip. In this system the increase of intensity with greater thickness of the paraffin oil is much larger than in the experimental case, and hence measurements can be made with much greater accuracy so that 20 A differences should be easily detectable. The cleavage step corresponding to the first maxima was observed, and by using Equation 1 its distance from the glass calculated. The nearest position of the mica could also be measured by using Equation 1. Then, by measuring the intensity change from either of these positions to the next cleavage plane, the height of the cleavage step could be calculated. The values were found to be multiples of 20 A, as expected for mica. In one instance, the nearest part of the mica to the glass gave a reflectivity of 0.00045 corresponding to 135 A separation from the glass, the next nearest step had a reflectivity of 0.00069 corresponding to 195 A; the difference corresponding to 3 mica sheets, the step next to this, had a reflectivity of 0.00121 corresponding to 295 A, 5 planes farther away. The system was also checked by counting the number of orders between top and bottom of the mica sheets (each order equals 1900 A) and comparing this thickness with that obtained independently by microscopic measurement. Sheets were between 1.9 and 4.0 micra thick. Good agreement was found.

For measurements on cells, the values of n_0, n_1, and n were found as follows. The value of $n_0 = 1.515$ (for the coverslip glass) is fixed, and values of n, the refractive index of the cell surface, were obtained from measurements by surface contact microscopy. For cells in normal medium, $n = 1.370$; after osmium tetroxide fixation, $n = 1.371$; after treatment with cupric ions, $n = 1.370$; and after treatment with 3 M NaCl, $n = 1.375$. These measurements show that the treatments have little effect on values of n. With the exception of 3 M sucrose treatment, there is little reason to suppose that the other media would appreciably alter the surface refractive index. The refractive index of the gap between cell and substrate, n_1, will be either that of the immersion medium or slightly greater because of the presence of intercellular material in the gap. For each measurement, n_1 has been taken as equal to the refractive index of the immersion medium since, as explained later, it is impossible to reconcile measured reflectivities with values of n_1 greater than 1.342 (except for 3 M NaCl or sucrose treatments which are special cases). The refractive indices of the various media in which the cells lay were measured in a refractometer and ranged from 1.338 to 1.341, except for the 3 M NaCl and sucrose media which had refractive indices of 1.360 and 1.420, respectively. Thus we can take $n_1 = 1.340$, except for 3 M NaCl and sucrose, in which n_1 has the values just given.

Thus a series of values can be fitted to Equation 1

to allow its solution in terms of d for the various measurements of reflectivity before and after the varying treatments of the cells. In Fig. 3, curves of the relationship between reflectivity and separation are shown for all treatments, including the two special cases of treatment with 3 M NaCl or sucrose. Calculation of model examples of equation 1 for a variety of different values of n and n_1 showed that only a small range of values (n, 1.365 to 1.380; n_1, 1.335 to 1.342) on either side of those used will give reflectivities similar to those measured over the range large (>200 A) to very small gap thicknesses (0 A).

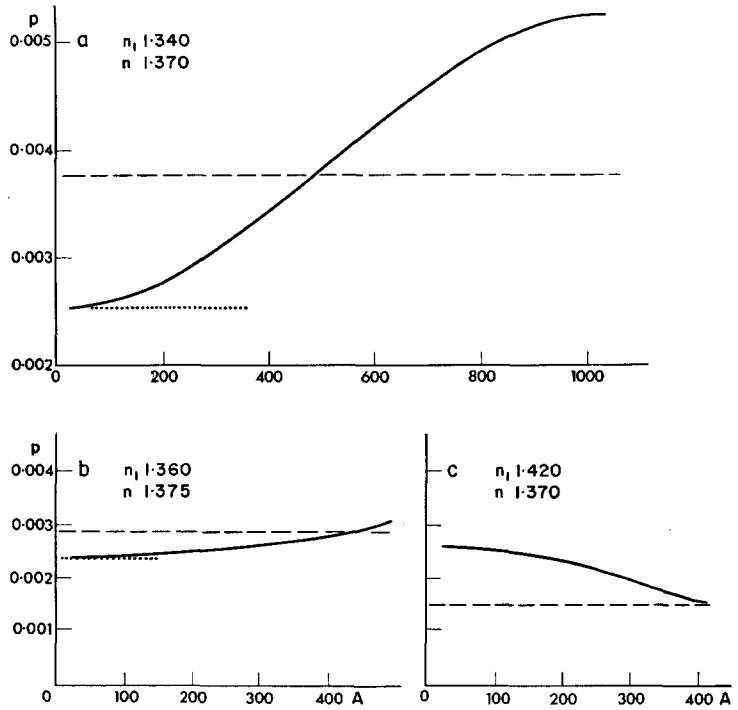


FIGURE 3 The relations between the reflectivity ratio p from an interphase and the thickness of the interphase in A for (*a*) cultures in all media save (*b*) cultures in 3 M NaCl, and (*c*) cultures in 3 M sucrose. n_1 and n for each curve are the values for interphase and cell surface refractive index, respectively. The broken line in each graph indicates the background reflectivity; the dotted line in *a* and *b* gives the lowest reflection ratio measured on any cell in each of these media respectively.

The problem of measuring the reflectivity was solved in the following manner: the reflectivity p at a single interface between two media is given by the equation:

$$p = \left(\frac{n_1 - n_0}{n_1 + n_0}\right)^2 \qquad (2)$$

where n_1 and n_0 are refractive indices of the two media. By preparing glass-water, glass-paraffin oil, glass-culture medium and glass-air interfaces and examining them, it was found that the ratio of measured reflection intensities for any pair of these interfaces was the same as that between the calculated reflectivities. Thus the intensity of the incident beam can be calculated, using Equation 2. The ratio between the measured reflection from part of the double interface over a cell and that from part of the glass-medium interface nearby, which can be calculated from measurements on a negative, allows the computation of the actual reflectivity of that part of the cell surface relative to the intensity of the incident beam. This reflectivity ratio can be used to evaluate the thickness of the thin gap between cell and glass by use of Equation 1.

The question remains, however, whether the inner side of the cell surface or constituents farther within the cell are able to reflect sufficient light to invalidate measurements.

The experimental test of this was to observe cells in white light. If colored interference bands are observed, these represent orders produced by thicknesses greater than *ca.* 2000 A. Consequently, reflection from the far side of the cell would be ex-

pected to produce colored fringes. In white light, all the interference fringes are zero order ones (black-white), except for faint colored ones over the nucleus and occasional colored fringes at the edges of the cells. Thus components deep within the cell or at the far side of the cell do not contribute to the interference pattern, and the fringes observed are produced by the gap alone. On theoretical grounds, Vasicek (reference 6, chap. 4) gives formulae for the calculation of reflectivity from a four-component system containing two apposed thin films. Calculation of the expected reflectivity for such a system which can be taken to represent glass, interphase between cell and glass, cell surface, and cell interior, using n_0 glass = 1.515, n_1 interspace = 1.340, n_2 cell surface = 1.370, and n cell interior 1.360 to 1.390, shows that these values are little different from those computed for the three-component system. Furthermore, it is possible that there is a gradient of refractive index away from cell surface; if this is so, reflection from the inner side of the cell surface will be diminished. Barer (13) immersed cells in protein solutions of high refractive index and observed that the interface between cell and medium became invisible with immersion media of refractive index *ca.* 1.36 to 1.38, which implies that the cell interior has a refractive index fairly close to that of its surface. In addition, it may be remarked that at no time has any sign of internal structure of the cell been seen under this form of microscopy, other than occasionally a patch representing the nucleus. A final reason for supposing that the measurements reflect accurately the thickness of the gap between glass and cell is that on raising the refractive index of the immersion medium by addition of 3 M NaCl or sucrose the reflectivity changes in the manner expected if the inner components of the cell play no appreciable part in contributing to the reflectivity.

With all of these points taken into consideration, three separate forms of Equation 1 have been used to calculate the thickness of the interphase from reflectivity measurements. The first equation for cells in all media, save 3 M sucrose and NaCl, has values n_0 = 1.515, n_1 = 1.340, n = 1.370. For 3 M sucrose, n_1 1.420, n_0 = 1.515, n = 1.370. For 3 M sodium chloride solution, n_1 = 1.360, n_0 = 1.515, n = 1.375. Curves of these three equations are shown in Fig. 3. Examination of the curves indicates that they flatten out below 100 A film thickness, which is why precise measurements cannot be made much below this limit, though it is possible to state that a given measurement of reflectivity gives a thickness of 75 A, or less than 50 A.

Accuracy of the Method

Inspection of Equation 1 shows that small changes in the value of n_1 and n lead to appreciable changes in the reflectivity. Although measurements of n_1 and n have been made to ± 0.001 refractive index unit, an accuracy which would limit the accuracy of measurements to ± 20 A, the values of n_1 have been chosen as being those of the refractive index of the bulk of the immersion medium. Values of n_1 might be inaccurate because of the presence of organic colloids lying in the gap between the plasma membranes, but the discussion will show that it is improbable that appreciable amounts of such materials are present. A low concentration, say 2 per cent w/v, of intercellular material such as mucopolysaccharide would have so small an effect on values of n_1 (an increase of *ca.* 0.003 refractive index units) that it would be without effect on the measurements. A set of refractive indices must apply in the system such that the lowest reflectivity found indicates a thickness greater than or equal to 0 A; this is satisfied with the values of n_1 and n used for the respective treatments.

A second source of inaccuracy may reside in the photographic and densitometric techniques. Slightly differing exposure times or development times may alter the over-all densities of the plates; however, the calibration curves (over the density range used) were such that the same ratio between background reflectivity and that of some part of a cell is preserved with small alterations in exposure and development. Since each plate possesses its own reference measurement, *i.e.* that of the background (medium-glass interface), small differences in exposure and development can be ignored since the calibration curves automatically correct such errors. Lastly, there remains the question of the accuracy of densitometric measurements, partly considered earlier. Diffraction effects are unlikely to be of importance if measurements are made on areas of the negative representing areas of the object greater than 2 micra wide: in consequence, all measurements were made on parts of the negative representing object portions of constant density 2 micra or more in width. The actual negative densities varied from 0.65 to 0.95 (except for cells treated with 3 M NaCl, in which the range was from 0.70 to 1.25). Within these ranges, densitometric readings were reproducible to less than 0.005 units, which correspond to an accuracy of reflectivity ratios within ± 2 per cent. In conclusion, it seems probable that reflectivity ratios can be measured with about 2 per cent accuracy and that although the accuracy of translation of these into interphase measurements varies with the interphase thickness itself and with the values of n_1 and n chosen, there is unlikely to be greater error than 50 A in such measurements for low values of d; these errors will be smaller for larger values of d. Even if the refractive index values are viewed with great scepticism, it will be admitted that changes in such

measurements after a cell has been treated with a reagent can be taken to indicate that the separation of the cell from the glass has changed.

RESULTS

Cells in Normal Culture Medium

Fifty individual cells in 46 cultures have been photographed by this method of microscopy. In all cases, as in other treatments, cells near the periphery of the outgrowth were examined in order that a portion of the background could be photographed with the cell and so that the edge of an individual cell could be easily distinguished. A further reason for doing this lies in the fact that it is thought by Abercrombie and Ambrose, (14) that the most adhesive part of the cell is its pseudopod and in consequence this part might be expected to adhere most closely to the substrate; cells with well developed pseudopods spread over glass are found only near the edge of the outgrowth.

In Fig. 4 three photographs of such cells can be seen. By making numerous transects of the negative with the microdensitometer, contour maps of the separation of the cells from the glass can be built up (see also Fig. 5). These photographs and maps illustrate a series of findings which could be made out in all the photographs of the cells. The greater part of the cell body lies 300 to 500 A away from the glass; this includes the centre part of the cell. The front pseudopod and, to a lesser extent, the rear pseudopod are those parts of the cell closest to the glass; large areas of such pseudopods are about 200 to 250 A away from the glass, and in many pseudopods small regions reaching a nearness limit of 100 A can be found. Frequently these 100 A regions form narrow bands either very close to the front edge of the cell or a little farther back but parallel to the leading edge of the cell. In most cells the rest of the cell body has a narrow band of separation 100 to 200 A, running around the edge of the cell, but in some very elongate cells with well developed pseudopods this band is missing, presumably because the stretching of the cell has pulled the whole of the centre part of the cell farther away from the glass. This bounding band can be seen in Fig. 4 and in Fig. 5 in a transect of a cell. Over the main part of the cell body one or other of two main patterns of separation develops. In many cells, including the obviously elongate cells, the surface is folded into a series of parallel furrows and ridges which are about 2 to 5 micra wide, the ridges of the cell coming to within 250 A and the furrows being as far away as 500 A from the glass. Examples of such cells can be seen in Fig. 4. The axis of this furrowing is in the direction of the movement of the cell, but it never extends into the pseudopodal region. In other cells a much more confused pattern develops (see Fig. 5): here, furrows and ridges are arranged apparently without order and merge into narrow pseudopods; nevertheless, the separations between the various parts of cell and the glass are within the same range as for the previously described type. This pattern develops in cells of a more rounded form but has never been seen in elongate cells. When the cells move, small changes in the pseudopods appear as a sort of slight dappling of light and dark as they extend. As the rest of the cell moves, the pattern of furrowing changes slowly but does not greatly alter in periods of 10 minutes. The background is

FIGURE 4 Interference reflection microscopy. Parts *a* and *c* are photographs of normal chick heart fibroblasts in normal culture medium. Note that the contrast ratio in these and all other photographs has been deliberately greatly exaggerated in preparing prints in order to make them suitable for reproduction. The original negatives appear as close grey tones. Parts *b* and *d* are "contour" maps of the interphase thickness between glass and cell prepared for the cells shown in *a* and *c* by densitometric transects on the negatives. Contours, *i.e.* isopachytes, at 100 A (not present), 150 A, 200 A, and 600 A. Areas >600 A away from glass indicated by dotted tint. Cell in *a* shows parallel lineation of regions of closest approach; only mid-part of cell body is shown. Note that centre of cell body is far away from glass. Two cells in *c*; cell at left has marked bounding band of adhesion; cell at right shows a more confused pattern of adhesion. A certain amount of fine detail has been ignored in production of the maps.

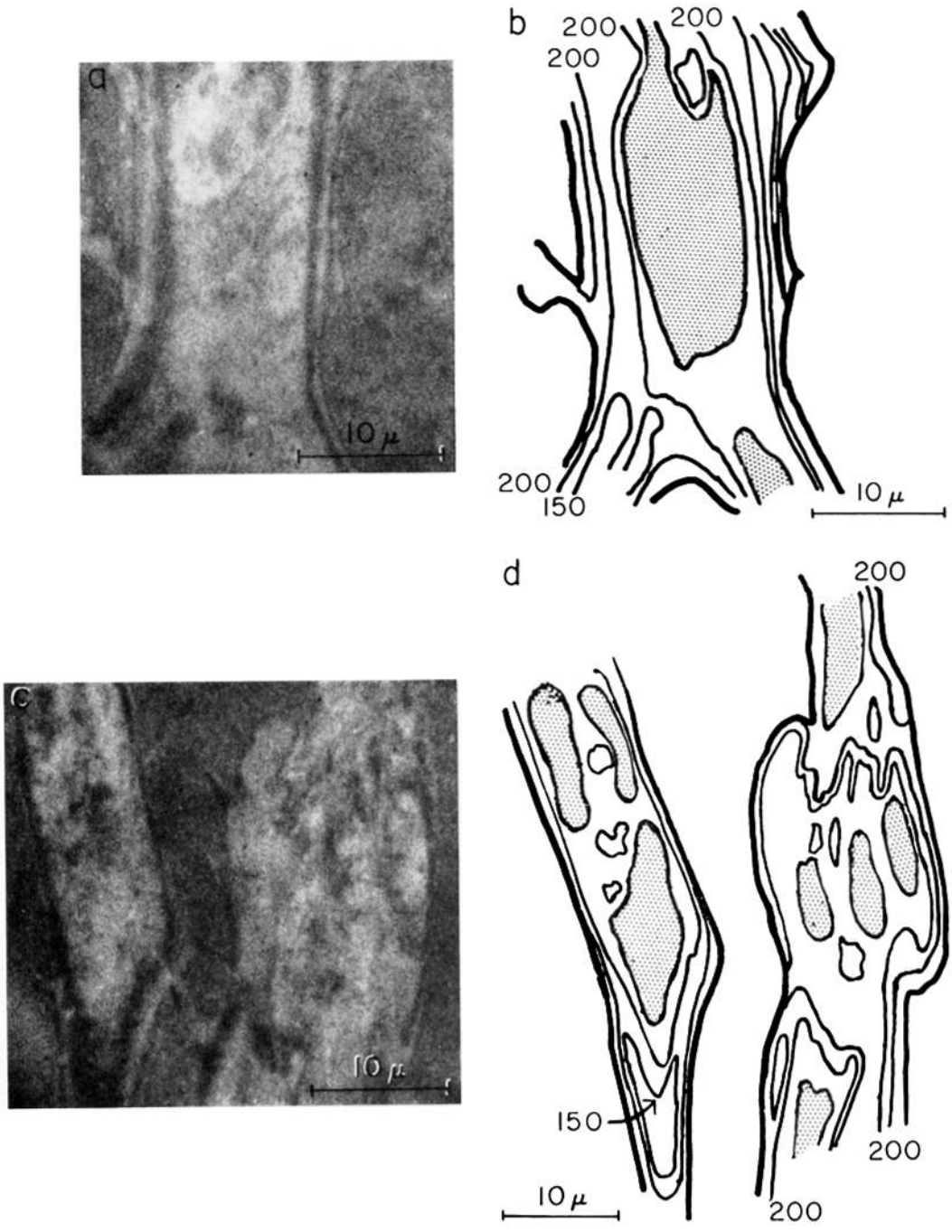
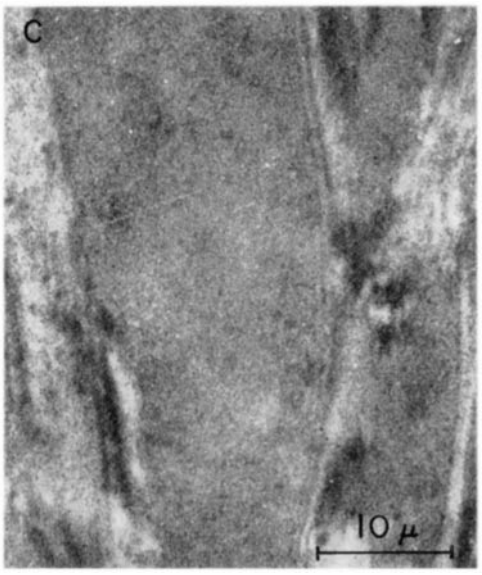


FIGURE 4 *a–d*



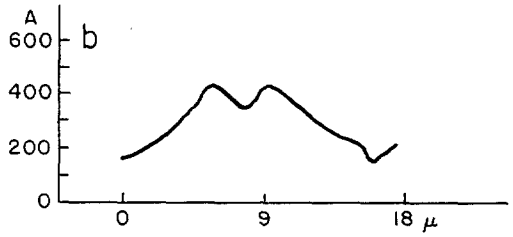


FIGURE 5 Fibroblasts in normal culture medium. *a*, fibroblast showing one marked region of adhesion near an edge; note that most of cell body is *ca.* 500 A away from the glass. *b*, section through cell-glass interphase, across another cell, to show edge adhesion; prepared from densitometric transect. *c*, on the left, a drawn-out cell showing region of adhesion near bottom of picture; on the right, region of junction between two or three cells, several triangular areas of adhesion close to contact of pseudopods.

of even density right up to the apparent edge of the cell, which suggests that the cells are not secreting any appreciable amount of material of high refractive index. Furthermore, the region from which the cells have just removed appears exactly similar to the background, which provides evidence that the cells do not leave behind an adhesive material. In 16 of the cells the region of closest approach of cell to glass was 100 A deep, in 20 it was between 125 A and 200 A, and in 14 cells it was between 200 A and 250 A.

Treatments which Decrease the Separation between Cell and Substrate

OSMIUM TETROXIDE FIXATION: Six cells from 5 cultures have been examined 2 minutes after fixation. The same cells had been photographed shortly before fixation and, although some changes were observable, in general terms they appeared much the same after fixation as before. Fig. 6 *a* shows the general appearance of such a cell. Transects (see Fig. 6 *c*) show that the closest approach of cell to glass is about 100 A

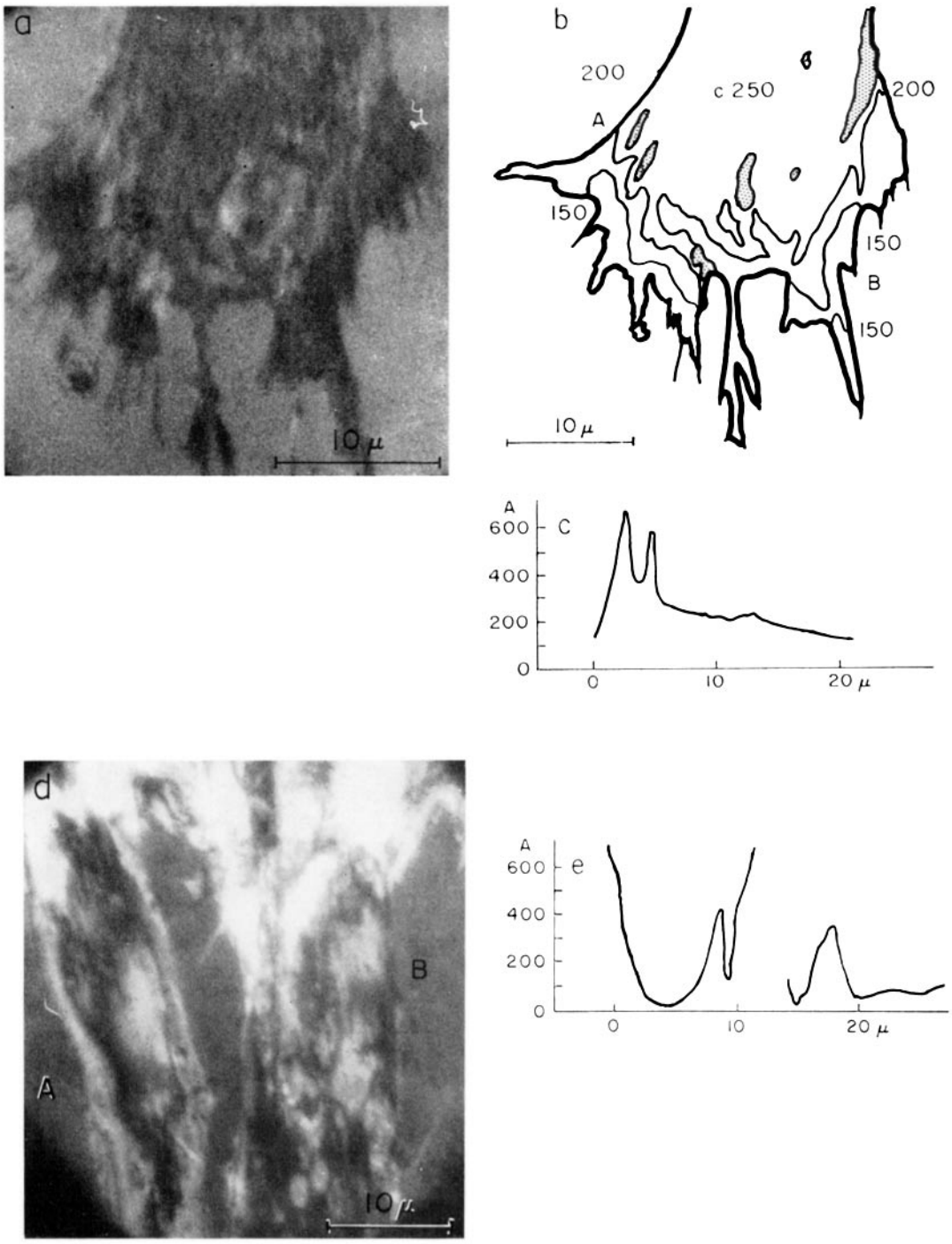


FIGURE 6 Osmium tetroxide and hypertonic NaCl treatments. *a*, cell after fixation with osmium tetroxide; pseudopodal region of cell. *b*, contour; *i.e.*, isopachyte map of interphase thickness for cell in *a*. *c* section through cell-glass interphase for cell in *a*; section runs from *A* to *B* (see part *b*). Note that osmium tetroxide fixation brings much more of the cell closer to the glass than it was before fixation, but that the cell is never closer than 100 A. *d*, two fibroblasts after treatment with 3 M NaCl; note the disorganised nature of the cells, but their outlines are still visible. *e*, section from *A* to *B* in *d*. Many regions of close, *i.e.* 50 A, adhesion form. Conventions for maps as in Fig. 4.

(found either in the band bounding the cell or in the pseudopod). The main mass of the cell body shows much less sign of furrowing than before treatment, but it remains about 200 A away from the glass. Visual observation of the cells during the addition of the osmium tetroxide reveals a very interesting phenomenon. The cells darken considerably a few seconds after the fixative is added. A few seconds later, the cells lighten again and their density reverts to roughly what it was before treatment. Unfortunately this phenomenon cannot be filmed because the light intensity is too low. Nevertheless this darkening implies that the cells come considerably closer to the glass during the process of fixation than they were before. But the cells then lift off the glass again and reassume roughly their former separation. The closest approach of osmium tetroxide fixed cells (in the steady state) to glass was 100 A in two cells, 150 A in three cells, and 250 A in one cell.

0.003 M CUPRIC CHLORIDE (UNBUFFERED): Cultures were injected with 0.003 M cupric chloride in 0.050 M NaCl: the pH of this medium varied from 4.8 to 5.4. Attempts to buffer this medium at pH 6.6 with Tris-HCl buffer were useless because a floc of fine precipitate formed, probably of cupric hydroxide, which resulted in messy pictures. Wilkins *et al.* (1) used unbuffered cupric chloride in order to avoid possible secondary effects due to the presence of buffer ions. Six cells were photographed after treatment. Transects of the negatives showed that the closest approach of cell to substrate was less than 50 A in all six cells, but that large parts of the cells remained 200 to 300 A away from the glass surface.

MEDIUM BUFFERED AT pH 3.6: Six cells from 6 cultures were photographed 2 minutes after the cultures had been injected with an 0.02 M acetate-HCl buffer pH 3.6 made up in 0.050 M NaCl. After this treatment the cells appeared rather ragged and it was obvious, by comparison with photographs of cells previous to treatment, that considerable shrinkage of the cells had occurred. Densitometric transects of the images revealed that the separation between cells and substrate had changed considerably. In the pseudopodal regions large areas were found in which the separation was less than 50 A, in other parts of the cells separations up to 300 A could be found.

3 M SODIUM CHLORIDE: The injection of 3 M NaCl pH 7.0 into cultures has dramatic effects on the cells. Although shrinkage of the whole cell occurs, the most obvious effect is a sharp rise in the image contrast (see Fig. 6). This appears to be due to much of the cell being distorted and pulled well away from the glass surface. Distances of 1000 A are found in many parts of the cells, such regions being found chiefly near the peripheries of the cell, but towards the centre of the cell a very different change occurs for large areas of the cell come closer than 50 A to the glass (see transect in Fig. 6). In all, seven cells from as many cultures have been examined

0.10 M CALCIUM CHLORIDE: This concentration of calcium chloride (made up in 0.050 M NaCl) buffered at pH 7.0 was chosen since it should bring the cells near to their point of charge reversal (1), in consequence suppressing their repulsive forces so that the separation between cell and substrate would diminish. Six cells from four cultures were photographed after injection. The transects of the negatives showed that large areas of the cells came to within 50 A of the substrate, but the folded topography of the interphase remained so that some parts lay as far away as 350 A.

Treatments which Increase the Separation between Cell and Substrate

0.050 M sodium chloride buffered at pH 8.20 appeared to have little effect on the cells. Densitometric transects of the images of four cells (the transects were chosen to cross those portions of the negatives which indicated closest approach of cell to glass) revealed no separation less than 225 A. Parts of the cell surface farthest away from the glass lay at a distance of about 600 A. Thus there appears to be an increase in the separation after treatment with this medium.

Three cells from three cultures were photographed 2 minutes after treatment with EDTA pH 8.20. Transects of the cells (16 in number) showed that no interphase distance less than 300 A could be found, and that much of the cell surfaces lay 600 A from the glass. This treatment failed to dissociate cells completely from glass.

Injections of either trypsin (0.5 per cent w/v solution in calcium- and magnesium-free culture medium) or distilled water were made into the cultures, but measurements of their effects were impossible because all the cells de-adhered from the

glass within 1 minute. It is of interest that no portion of the cell, such as a small piece of pseudopod, was ever seen to be left behind

Treatments which Do Not Affect the Separation

After injection of 3 M sucrose the contrast of the image falls considerably because the high refractive index of this medium reduces the contrast expected from a given separation of cell and substrate. But the curve relating image density and separation is of much the same degree of curvature, though in the opposite direction (see Fig. 3), as curves for other treatments, so that measurements are nearly as accurate. On the five cells treated from five cultures, densitometric transects have shown that the separation between cell and substrate is never less than 100 A though frequently much greater (*e.g.* up to 600 A). It is obvious also that this treatment considerably distorts the cells.

DISCUSSION

The various treatments made on the cells adhering to glass show that the distance between plasmalemma and the glass substrate can be altered with ease. Presumably the manner in which the interphase distance can be altered will be strongly indicative of one or other mechanism of adhesion and this point will be explored. However, one problem must be resolved before this can be done. The observations show that the interphase distance alters from one part of the cell to another. Although some treatment may considerably alter this distance in one small part of the cell, its effect over the whole of the cell may be so slight that the percentage change will be insignificant. Indeed the results suggest this. For example, though treatment at pH 3.6 makes small parts of the cell close down to an interphase distance of less than 50 A, much of the cell is either unaffected or may show increased interphase distance. There are strong reasons for thinking that the significant distance is the closest one. The main reason is that when the adhesion of the cell has come to equilibrium, the distance between cell and substrate will be one at which the forces of adhesion and repulsion come to equilibrium, whatever the mechanism of these forces. Obviously the farther away cell and substrate are (beyond a very close minimum) the weaker the adhesion will be. In consequence it seems that the point of closest approach will be the point of cell adhesion or at least of strongest adhesion. A biological reason for choosing these sites for investigation of the mechanism of adhesion is that they are found generally in the pseudopodal parts of the cell which are thought (5, 14) to be the most adhesive regions.

In the Introduction, the three main theories of cell adhesion were outlined, namely (1) that there is a gap between the cell surfaces, the gap being filled by a substance binding the two plasmalemmata together by chemical bonding, (2) that there is no gap, the plasmalemmata binding directly to one another, by close-range van der Waals–London forces or by chemical bonds, and (3) that there is a gap between cell surfaces, the main adhesive forces being the van der Waals–London long-range forces, the electrostatic forces of repulsion due to the surface charges balancing with them to give a separation of *ca.* 100 to 200 A between cells (see references, 4, 15). Obviously, the second and third theories predict that adhesions form with a gap of 200 A or less between the cells, but it is harder to suggest what gap distance would be predicted by the first theory. Indeed the comparative constancy of the 100 to 200 A gap between cells seen in electron micrographs would seem to require some special and unsuspected property of a cement substance if no artefacts are formed on fixation and if theory 1 is correct.

I intend to discuss the results in terms of their applicability to theory 3, which has been described in detail in a previous communication (4). Van der Waals–London forces are little affected by chemical treatments, but the surface potential of the cells can be considerably affected by the changes in cation valency, ionic concentration, pH, etc. Osmotic changes unaccompanied by alteration of the ionic concentration have no effect on the surface potential. These two conditions may form the experimental crux upon which the accuracy of theory 3 can be tested. Any deviation of cell separation distance in adhesions or of adhesive behavior from those expected, if they are directly controlled by the surface potential, will suggest that some other mechanism is acting, perhaps involving specific chemical bonding. The various treatments used were chosen partly because of their known effect on surface potential and partly because some of them would be expected to have contrary effects if such adhesive systems as specific chemical bonding do act.

When the electrostatic repulsive forces are reduced, for instance, by increasing the cation concentration or valency, or by lowering the pH towards the isoelectric point of the cell surface (charge reversal point), the repulsive forces decrease, and in consequence the potential energy barrier is sufficiently low for the two surfaces to approach to *ca.* 10 to 20 A, where close-range van der Waals–London forces can act. In the present series of measurements, treatments with 3 M NaCl, or 0.10 M $CaCl_2$, or 0.003 M $CuCl_2$, or pH 3.6 treatments would be expected to have such effects, and the measurements show that indeed they do reduce the distance of closest approach to below 50 A. Since the form of the potential energy curve of repulsion suggests that it is extremely unlikely that adhesions would form with a gap distance of *ca.* 30 to 70 A, measurements of gap thickness less than 50 A can be taken to mean thicknesses below 30 A; *i.e.*, in the close or primary range of adhesion.

By increasing the repulsive forces, the gap thickness can be enlarged. Treatments which can be expected to bring this about, *e.g.* lowering the ionic concentration by treatment with distilled water, raising the pH to 8.22, removing divalent ions by treatment with EDTA, have been found to increase the nearest approach of cell and substrate to 200 A.

These results are compatible with the third theory. But although the finding that in normal culture medium the nearest approach of cell and substrate is never less than 100 A is directly contradictory to theory 2, these results are in general in agreement at first sight with a variant of this second theory. The variant theory maintains that the charged groups of the cell surface provide repulsive forces but that the main attractive forces are brought about by the combination of calcium atoms with carboxyl groups on the cell surface, the individual calcium atoms attaching to the carboxyl groups on one cell surface and to other carboxyl groups of some macromolecular substance (cement) found in the gap between the cells. At the other side of the gap, other calcium atoms bind the molecules of cement to the surface of the other cell (16, 17). If this theory were true, it would be expected that on reducing the pH the ionization of carboxyl groups would die away and that the calcium links would be broken, thus removing the main attractive force. Yet the results show that at pH 3.6 (a degree of acidity sufficient to suppress carboxyl ionization) the cells form closer adhesions with the substrate than at higher pH, and though in part this close approach must be due to the suppression of the surface charge of the cell, an attractive force must still exist at this pH for the adhesions to form. This force cannot be due to the existence of calcium-carboxyl links. Steinberg (18) has claimed that the aggregative behavior of amphibian embryonic cells can be equated with their adhesiveness and that they do not aggregate below pH 4.5. However, his conclusion that aggregation is equivalent to adhesion is questionable (see reference 4), and Curtis (19) finds that embryonic chick and amphibian cells will adhere at pH 4.0.

Consider further the possibility of the action of a cementing substance. The results show that such a material, if present, can be compressed in thickness from 200 A to less than 50 A, probably to 10 A; can contract under the influence of low pH, calcium ions and high ionic concentration; can expand under the influence of lack of Ca ions, high pH, and low ionic concentration; and yet is unaffected by purely osmotic phenomena such as the addition of 3 M sucrose solution. This hypothetical cementing substance has, in fact, to respond to these various treatments in exactly the same manner as the surface potential, and in addition must be capable of an improbable degree of shrinkage (20).

No evidence for the existence of a cementing material has been found from the measurements with interference reflection microscopy. Although a refractive index of $n_1 = 1.340$ has been used for the interphase medium in the calculations, this value was chosen because it is the refractive index of the medium at 36°C. The medium contains approximately 6 per cent protein. At first sight it might be possible to explain that in the interphase in normal medium the serum protein is replaced by a cementing substance of similar refractive index. However, when this gap is shrunk to at least a quarter of its normal thickness, as at pH 3.6, the concentration of the hypothetical cementing substance would rise fourfold. In consequence the refractive index of the interphase medium would rise to *ca.* 1.367 (assuming a refractive index increment equal to that for protein). If this value existed, the measured reflectivities of up to −33 per cent below background would be impossible, as has been mentioned earlier. It can be concluded that the results do not

suggest that a cementing substance acts in the adhesion of these cells to glass. Of course, this does not preclude the existence of intercellular materials that play some part in the adhesion of other cells in other situations.

Wilkins *et al.* (1) claimed that the flocculation kinetics of polymorph leucocytes treated with cupric, lanthanic, or thoric ions were such that the cells must come into close contact; *i.e.*, with a gap of *ca.* 10 to 20 A between cells. This result would in any case be expected, since these ions, in the concentrations used, considerably diminish the electrostatic forces. These authors argued that when cupric ions were used the cells remained alive and hence that these systems form reliable models for cell adhesion in normal physiological media. Since only slightly stronger cupric salt solutions have been used as fixatives (21), it seems improbable that the cells were alive.

It would be expected that osmium tetroxide fixation would reduce the gap between two surfaces because it would suppress their negative charges. Yet this effect is not observable in the photographs, but visual observation suggests that a transitory reduction of the gap occurs. At present it is only possible to speculate on the mechanism of this phenomenon, which requires confirmation. It may, however, be that osmium tetroxide fixation produces a complete reversal in charge because of the comparatively high concentration of osmium tetroxide, so that the cells re-separate as the membranes become positively charged. In any case, the phenomenon is of the greatest interest to electron microscopists, since it seems possible that the 100 to 200 A gap found between fixed cells is an artefact, but an artefact which fairly closely reproduces the situation in life.

The osmotic shock of 3 M NaCl or sucrose treatments in the present work might have produced meaningless measurements. This seems unlikely because the cells have adhesions only on one side. Shrinkage or expansion of the cells due to osmosis will produce only lateral pulls on these adhesions and, though some adhesions may be destroyed, no force of osmotic origin will tend to compress existing adhesions.

It may at first sight seem surprising that trypsin apparently is able to affect the repulsive forces so that de-adhesion occurs, for no cement appears to be present which it might dissolve. However, as Seaman and Heard (22) and Heard and Seaman (23) suggest, trypsin may affect the surface potential of a cell.

It can thus be seen that the effects of various physicochemical conditions on the distance between cell and substrate are exactly those predicted if van der Waals–London forces and electrostatic repulsive forces act between the surfaces to determine their adhesive nature (with the possible exception of evidence from trypsin treatment). Although this view has been advanced on previous occasions (Curtis, 4, 5), experimental evidence in support of this mechanism has been lacking.

This interpretation of the results applies, of course, only to cell-to-glass adhesion. It is unclear at present whether a universal mechanism of cell adhesion exists irrespective of whether the substrate is another cell or a non-living structure. Berwick and Coman (24) suggest that cell-to-cell adhesion differs fundamentally from cell-to-glass adhesion, but theoretical studies (4, 5) and practical investigation of fibroblast adhesion (25, 26) imply that the two situations may be almost identical as regards adhesive mechanism.

Weiss (17) has proposed that, when adhesions between cells and their substrates are broken, the line of rupture may not always pass through the gap between plasmalemma and substrate, but that it will often run so that small portions of the cell are left on the substrate. No evidence for such a phenomenon has been found when the sites once occupied by cells have been examined (after their de-adhesion) by interference reflection microscopy. It is possible that the large shearing forces which Weiss used to remove his cells from the glass substrate tore the plasmalemma, thus leaving pieces of cell behind, whereas in the present work the only force applied to cause de-adhesion was the weight of the cell pulling under gravity.

De-adhesion of cells, treated with trypsin or distilled water, took less than one minute. I have suggested (5) that if the 100 to 200 A gap, apparent from electron micrographs, extended over large areas of the contact between two cells, then it would be expected, for mechanical reasons, that it would take *ca.* 10^4 seconds for this gap to be increased from 100 A to 1000 A (for a contact area of *ca.* 300 μ^2): this was termed the 'drainage' problem. However, experimental observations show that de-adhesion is much more rapid. It seems that the reason for this rapidity of de-adhe-

sion lies in the fact that only the edges of the cell come into the 100 A adhesion with the substrate. The time for separation is proportional to the area of contact and to the inverse square of the distance of separation: in consequence a small area of 100 A adhesion accompanied by a large area of *ca.* 300 to 400 A gap can be separated to a distance of 1000 A much more rapidly than a large area of 100 A adhesion. Thus it can be appreciated that the physical considerations involved in the rate of de-adhesion (and consequently adhesion) are not contradicted by the experimental results, and indeed the rate of de-adhesion indirectly confirms that only small portions of the surfaces of these cells can be as close as 100 A to the substrate. In addition, this suggests that the large regions of *ca.* 100 to 200 A gap seen between certain cells in electron micrographs, e. g. liver cells (27), which cells are easily dissociated by chelating agents (28), may be partially artefactual, an idea borne out by the measurements made on osmium tetroxide fixed-cells.

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