SUMMARY

Using both living and glutaraldehyde-fixed red cells, we have examined adhesion to both oil/saline and glass/saline interfaces by interference reflection microscopy. At low ionic strength, 0.4 mM NaCl, fixed cells adherent to the oil/saline interface show first order whitish yellow zones of closest approach which indicate a separation of ~100 nm. Quantitative interferometry in monochromatic light supports this conclusion. As the salt concentration is increased the separation decreases and the final image shows zero-order black which probably indicates molecular contact with the interface. Similar but less reproducible results were obtained with fixed and unfixed cells on glass. These observations show that physical interactions alone can be responsible for adhesion in dilute and concentrated salt solutions. It is not, however, believed that the results necessarily imply the existence of adhesion with a gap in physiological concentrations of salt.

INTRODUCTION

We have described several experimental techniques for examining the adhesion of human red cells to clean surfaces (Gingell & Todd, 1975; Todd & Gingell, 1980; Gingell & Fornés, 1975, 1976; Gingell, Todd & Parsegian, 1977). The results of these experiments have been discussed in relation to physical forces of long-range attraction and repulsion (Parsegian & Gingell, 1973, 1980). Here we produce interferometric evidence which supports our prediction that, in sufficiently dilute saline, adhesion can take place with a gap of ~100 nm between cell surface membrane and substratum. For well-defined interfaces of the types we have used, this mode of adhesion can be adequately demonstrated in dilute salt solutions where the Debye length, and hence the electrostatic repulsion governing the gap, is large enough to give an unambiguous first-order very pale yellow interference image. The technique used involved sedimentation of glutaraldehyde-fixed red cells on to a flattened hexadecane/saline interface in a closed chamber. After inverting the chamber the lid was removed and a 100 x microscope objective was immersed in the hexadecane: cells at the interface could then be observed at high magnification using an interference-reflection microscope. From the interference colour it is possible to make an approximate estimate of the distance of closest approach of the cells to the oil/water interface. It has also been possible to use a recently developed quantitative photometric technique for measuring cell-substratum separation (Gingell & Todd, 1979) despite Brownian motion of the red cells. This method has also been applied successfully to Dictyostelium discoideum amoebae on glass (Vince & Gingell, 1979).
MATERIALS AND METHODS

Glutaraldehyde-fixed cells, electrolyte solutions and hexadecane were prepared as described earlier (Gingell & Todd, 1975). We used a Zeiss Universal microscope with a vertical illuminator type IIc, which has a field stop iris diaphragm, and a Zeiss planachromat 100 x objective. For video recording we used an aperture stop of 1 mm, corresponding to an illuminating numerical aperture (INA) of approx. 0.69 (see Gingell & Todd, 1979), but for quantitative work a 2.0-mm aperture was used in conjunction with green light (Schott type AL, 546-nm interference filter) from an Oriel 1000-W Xenon arc with a special condenser and heat filtration set up as described.

Photometric measurements were made as described previously (Gingell & Todd, 1979), using a 25-μm pinhole in the aerial image plane which receives light from an area of the object plane having a diameter of 0.2 μm. This equals the area of an object in the focal plane which is observed by the detection system. Computed separations derived from measured irradiances involved evaluation of an angular light distribution function which is described below. White-light images were observed and recorded, using closed circuit colour television. The equipment included a Hitachi model HV 1100 AU 3-tube colour camera with sensitive Chalnicon tubes, a Sony model VO 1810 video tape recorder and a Sony video monitor. Photographs were taken from the monitor using Ektachrome Professional 200 ASA reversal film and prints were processed using Ilford Cibachrome materials.

For white light observations the experimental chamber (Fig. 1) consisted of a base made of polytetrafluoroethylene (PTFE) and an upper segment of stainless steel (type EN 58-J which is salt resistant after passivation in 70% nitric acid). An accurately machined rod of this steel was passed through the PTFE segment: separate blind drillings in the rod held a concentrated suspension of red cells in distilled water and a concentrated saline solution. The bar was threaded at one end so that it could be drawn through the chamber whilst keeping vibration to a minimum. An EN 58-J encased soft iron bar for magnetic stirring was placed in the lower segment. Two volume-adjusting devices, each consisting of concentric screws and a silicone rubber diaphragm were located in the wall of the PTFE segment. For clarity these are shown in the same plane as the steel bar in the figure but in practice they were set at an angle to it. An O-ring of PTFE sealed the junction of the 2 segments when they were pulled together by setscrews, which are not shown. A PTFE lid was located on the steel segment by setscrews. The rod was positioned with both drillings in the PTFE wall. The PTFE segment was then assembled beneath the steel one and was filled with dilute saline, avoiding air bubbles. After filling the steel segment with hexadecane, the oil/water interface was sucked clean using a finely drawn Pasteur pipette and the interface was made flat with the screw adjusters. The PTFE lid was then put on and the bar was drawn through sufficiently and rotated through 180°, to enable the red cells to fall into the chamber. This method of addition obviated contact of cells with air/water interfaces. The cell suspension was gently agitated by revolving a magnet beneath the chamber, thereby rotating the enclosed soft iron bar. The chamber was carefully inverted, keeping the magnet in position (Fig. 1B) and left for 20 min for cells to settle on to the oil/water interface. After restoring the chamber to its original orientation and removing the lid it was placed on the microscope stage and the cleaned objective was immersed directly in the hexadecane and focused on the interface, making any necessary levelling adjustments to the interface with the screws. When it was required to increase the salt concentration the transverse rod was drawn further through the chamber so that concentrated saline was released, after which the solution was stirred again using the magnet. Cells at the interface were observed more or less continuously during this concentration increase as there was no volume change and hence no displacement of the interface. The final salt concentration was measured conductimetrically on a Wayne-Kerr 200 bridge after the experiment.

For photometry, the transverse rod was removed to reduce reflection, the holes in the PTFE chamber wereblanked off, and an anti-reflective platinum-blackened platinum disk was inserted into the base. Cells were in this case added by pipette through the oil/water interface before adjusting it for planarity.

Red cells attached to glass were viewed either in a simple coverslip/cavity slide preparation, or in a stainless steel chamber with a coverslip upper window and a non-reflecting platinum black base. The objective lens was oiled to the coverslip.
Cells, salt solutions, hexadecane and all glassware were cleaned and prepared as described previously (Gingell & Todd, 1975), taking great care to ensure that the oil/water interface was free of particulate or surface-active contamination. Poly-L-lysine treatment of glass was effected by exposing clean coverslips to 0.1% poly-L-lysine (Sigma, Type VIIb) solution for a minute, then rinsing well in 4-times distilled water.

Vibration was a potentially serious problem, as cells on the liquid interface were exquisitely sensitive to mechanical displacement. We overcame this difficulty by avoiding mechanical contact between lamphouse and microscope, and by mounting the latter on a 0.5-ton (0.51 x 10^3 kg approx.) stack of concrete blocks resting on a thick wooden base supported by large rubber bungs in a basement laboratory.

The refractive index of red cell cytoplasm was obtained by centrifuging saline-washed red cells at 6000 rev/min in a bench centrifuge until packing was constant. After removing the supernatant, packed cells were lysed by sonication using a Dawe Soniprobe, taking care to avoid overheating. Completeness of the process was checked by phase-contrast microscopy. A value of 1.393 was found using an Abbé refractometer.

**Fig. 1.** Inversion chamber. A, releasing cells into the chamber. B, chamber inverted: cells settle towards the oil/water interface. C, chamber righted: adherent cells remain attached to the interface. Components: L, lid; H, hexadecane compartment; HW, hexadecane/water interface; TN, threaded nut; P, PTFE section of body; SB, soft iron rod encased in stainless steel; M, round magnet; SD, silicone rubber diaphragm; AS, water volume adjusting screw; S, recessed well for concentrated saline; SR, stainless steel rod. In C, OB, microscope objective; H, hexadecane.
Optical weight function for objective immersed in hexadecane

The weight function \( W(\theta) \) describes the distribution of flux with respect to \( \theta \), the angle of incidence. This function was derived for a lens in immersion oil (Gingell & Todd, 1979), but a modification is required when the oil is hexadecane, which has a different refractive index (1.4345 compared with 1.515). The measured irradiance per unit area as a function of \( \theta \) in immersion oil was found to be \( I(\theta) = 0.875 \cos^4 \theta \) except at the edge of the cone of light, where irradiance fell sharply. It can be shown that in a medium of refractive index \( n \) this transforms to

\[
I(\theta) = 0.875 \cos \theta \cos^4 \left( \frac{n}{1.515} \sin \theta \right).
\]

(1)

Since the weight function is defined as

\[
W(\theta) = \frac{\sin \theta}{0.875 \cos^4 \theta} I(\theta)
\]

(2)

we have in medium \( n \)

\[
W(\theta) = \sin \theta \cos^4 \left( \sin^{-1} \left( \frac{n}{1.515} \sin \theta \right) \right) / \cos^4 \theta.
\]

(3)

The weight function at the edge of the cone of light is found to be \( W(\theta) = 5.84 - 0.104 \theta \) when \( \text{NA} = 1.2 \). (Fig. 2.) Calculation of cell-interface separation from measured irradiance was carried out by the procedure of Gingell & Todd (1979).

RESULTS

In 0.4 mM NaCl cells hanging beneath the interface showed a characteristic pattern of white-light Fizeau fringes localized at the cell-oil–water interface. Brownian motion of the adherent cells caused some flickering of the pattern, but the regions of closest approach showed as rather bright pale yellow regions surrounded by a

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Fig. 2. Weight function \( W(\theta) \) used in computation when using hexadecane as an immersion medium. Curve (a) is the weight function given by equation 3. Curve (b) is the function \( W(\theta) = 5.84 - 0.104 \theta \), which describes the fall off in flux near the edge of the cone of light.
Interferometry of cell-substratum adhesion

dark fringe with coloured edges, suggesting first-order interference (Fig. 3A, B). Up to 6 fringe orders were discernible. Cells adherent to the interface in an edge-on configuration showed a single small pale yellow point of closest approach. Sometimes Brownian movement perpendicular to the interface would cause the colour to oscillate between white and orange-red. A cell photographed at two stages of an oscillation sequence is shown in Fig. 3C, D. At higher salt concentration where the closest contacts were grey or black such oscillations were never seen. Further oscillation is shown in Fig. 3E, F, where the cell appears to be tilting about a small whitish-yellow area.

To ascertain the order of interference and thus the separation between cells and the oil–water interface in 0.4 mM NaCl we observed the fringe pattern as the salt concentration was raised from 0.4 to 72 mM. Continuous observation (recorded on videotape) showed that the region of closest approach exhibited a series of first-order colour changes, going first from whitish-yellow to silver-grey and then through darker grey to black. As the salt concentration increased a striking thing was seen: some cells which showed localized zero-order black regions (Fig. 3G) suddenly ‘snapped’ into a state where most or all of the coloured fringes disappeared (Fig. 3H). The image became grey and black, but sometimes retained a small coloured region. Occasionally 2 such cells would dart rapidly together over distances of several cell diameters, a phenomenon we also noticed in bright-field observations at concentrations ~ 10 mM NaCl, but not at 0.4 mM NaCl.

We also examined aldehyde-fixed cells on glass coverslips. In 2.0 mM NaCl adherent cells showed a fringe pattern (Fig. 3I) similar to that seen on oil at the same concentration. Cells in distilled water viewed in an inverted Zeiss interference microscope did not adhere to glass and could be seen to execute extensive Brownian motion perpendicular to the interface, causing the fringes to fluctuate through a whole order of interference. Physiological saline, 145 mM NaCl, resulted in a completely different pattern; cells remained motionless, showing very localized areas of highly characteristic zero-order black (Fig. 3K), whereas cells at an intermediate salt concentration showed a grey area of closest approach (Fig. 3J).

Fig. 4 shows experimentally measured relative irradiances superimposed on a computed curve of irradiance vs. cell-substratum separation for 2.0-mm illuminating aperture. The experimental values are of very low accuracy due to the difficulty of keeping erratically moving cells targeted on the photomultiplier pinhole. Average irradiances for 3 cells (only) in 0.4 mM NaCl were assessed from chart-recorder traces. We also measured the irradiances of several black areas of cells which had ‘snapped’ down on to the hexadecane-saline interface: the average value is also shown in Fig. 4.

Cells were observed on glass rendered positively charged with poly-L-lysine. Maximal attraction of cells which occurred in distilled water resulted in localized zero-order black contacts of sufficient strength to prevent Brownian motion.

We also examined live unfixed red cells in 290 mM isotonic sucrose with 0.5 mM NaCl. Unfixed cells are very sensitive to osmotic stress and care was taken to avoid evaporation from the edges of the coverslip preparation, since this caused a discocyte-echinocyte transformation. Cells which remained adherent in isotonic 0.5 mM NaCl usually
had pale-yellow closest-approach zones, though purple was also seen. Occasionally cells with extensive black contacts were seen—sometimes bursting and fading into pale-grey image. At a slightly higher salt concentration, 2.0 mM NaCl, living cells in 290 mM sucrose showed grey or black images: the latter appeared to be highly distorted, resulting in a maximal area of contact.

Limited observations were also made on living cells in the presence of serum protein, using ~ 0.1 percent total protein in 145 mM NaCl. Adherent cells showed grey closest-approach regions which, unlike grey areas of live or fixed cells on clean glass, were seen to roll around the periphery. We also exposed cells under similar conditions to glass pretreated with poly-L-lysine solution. The localized black contacts which formed were immobile, though the highly flexible cells oscillated about the contact points.

In contrast to the results obtained at the hexadecane/saline interface, red cell adhesion to clean glass coverslips has shown considerable variation over a period of several years which we have not been able to elucidate.

Fig. 3. A–H, reproductions from videotape recording of red blood cells adherent to a hexadecane/saline interface. Oil is above and water below; attached cells are hanging beneath the interface. INA = 0.69. A, B, 2 different cells in 0.4 mM NaCl. These cells showed no discernible oscillation perpendicular to the interface and remained adherent with a distance of closest approach ~ 100 nm, as judged by the large pale whitish-yellow region within the first-order dark band. Cell A shows one such region, cell B shows two. C, D, 2 stages in Brownian oscillation perpendicular to the interface exhibited by a cell with an end-on contact in 0.4 mM NaCl. The closest approach in C shows as pale whitish-yellow whereas the same region in D is more mustard yellow and a new region of closest approach has appeared on the left of the cell. The orientation of the cell is similar in C and D as can be seen on the original videotape record. E, F, 2 stages in Brownian oscillation in 0.4 mM NaCl, which has resulted in the whole cell tilting about a relatively stable closest approach region. This is the larger whitish spot inside the dark first-order fringe (arrows). The change in separation over the lower two-thirds of this cell is from ~ 320 nm (first-order yellow) to ~ 400 nm (first-order blue). A transient zero-order whitish region ~ 100 nm separation present at stage E is absent from F. The diameter of the arrowed zero-order region can be seen to oscillate in the videotape, indicating either a small transient deformation of the interface or a rhythmic approach and recession from the interface over this area. G, H, a pair of cells following concentration of the saline from 0.4 to 72 mM NaCl. No oscillation perpendicular to the interface was seen. In G both cells show grey/black regions of zero order contact with the oil/water interface (general area arrowed). In H the lower cell has snapped suddenly down onto the interface and the entire image has become black and grey. The upper cell followed suit soon after. × 5000 approx.

Frames I–K show cells adherent to a glass/saline interface, photographed directly. Glass above, water below. I, shows a cell in 2 mM NaCl. The closest contact is zero-order pale whitish-yellow indicating a separation of ~ 100 nm. Paler fringes arising from the far side of the cell are visible in this region. J, a cell in slightly more concentrated saline, showing a zero-order grey region of closest approach. K, a different cell in 150 mM NaCl showing zero-order black at the closest approach region, indicating ~ zero separation. × 5000 approx.
DISCUSSION

Our experiments using an oil/water interface lead us to 4 conclusions: (1) aldehyde-fixed red cells can adhere to an inert hydrocarbon interface by physical forces without specific biochemical interactions; (2) in dilute salt solutions cells can adhere to the interface with a minimum resolvable distance of separation ~ 100 nm; (3) as the salt concentration is raised the separation of the cell from the interface decreases; (4) at physiological salt concentration the interaction between the cell and the interface becomes qualitatively different and appears to be stronger than at lower concentrations. The reasons for these conclusions will now be discussed.

We have previously demonstrated that fixed red cells can adhere to a clean hydrocarbon interface (Gingell & Todd, 1975; Todd & Gingell, 1980; Gingell et al. 1977). We also found that red cells allowed to settle on a hexadecane/145 mM NaCl interface cause no change in the interfacial tension, showing that no measurable amount of protein or other surfactant is released by the fixed cells; consequently we have no reason to believe that cells adhere to macromolecules absorbed to the interface. Our result contrasts with that of Maroudas (1975) who reported that fibroblasts in serum-free physiological saline would not spread on uncharged paraffin wax. However, Vasiliev & Gelfand (1978) described the attachment and spreading of fibroblasts on uncharged lipid films below their transition temperature where the hydrocarbon chains are gelled, but noted a failure to spread above the transition temperature. It may be that fluid lipid and hydrocarbon interfaces in certain circumstances cannot
support the tangential stresses required for spreading, while exerting an attractive force sufficient to cause adhesion: in this case cells would remain rounded and weakly attached over a limited area. The area of passive contact without active cell shape change will depend on the relative sizes of the deforming energy, which is the attractive energy per unit area, and the resistance to deformation of both cell and interface. In physiological saline the interaction of unfixed red cells with the hexadecane interface is sufficient to rupture the cells; this indicates that they come into molecular contact with the oil, whereupon the surface tension is locally reduced by protein adsorption, leading to rapid expansion of the contact region and cellular rupture. In the experiment described by Rosenberg (1964) cells at a liquid fluorocarbon/saline interface were able to spread in the presence of serum protein. Protein adsorption performs 2 functions by reducing the interfacial tension; it protects cells from lysis and provides a rigid mat of denatured protein which supports tangential spreading stresses.

Our second conclusion, that red cells can adhere to the oil/water interface at very low ionic strength with a finite gap, was deduced from a theoretical analysis of earlier experimental work (Gingell et al. 1977; Parsegian & Gingell, 1980) and is verified by the present optical study. There are, however, 2 problems in image interpretation which affect the accuracy of distance estimates. Since the light illuminating the specimen converges on it from a cone, defined by the illuminating numerical aperture (INA), there will not be a unique optical path difference for light which recombines to form the interference image. This was discussed qualitatively by Izzard & Lochner (1976) and has recently been treated rigorously for monochromatic light by Gingell & Todd (1979). The fact remains that there is no quantitative theory for white light microscope interferometry. The best that can be done in white light is to make observations at small INA approximating to normal incidence for colour assessment (which is at best a subjective procedure) and then refer to a standard colour chart such as Zeiss S41-500-o-c. Taking an optical path difference (OPD) corresponding to the middle of the pale yellow (OPD ~ 300 nm) we obtain the separation $d = \frac{300}{2 \times 1.4345} \approx 100$ nm, assuming normal incidence.

Using our photometric method, we obtain a separation in 0.4 mM NaCl of ~ 120 nm (Fig. 4). The extreme technical difficulty of such measurements, due to the fact that cells on the interface execute Brownian motion in three dimensions, render this distance assessment imprecise. This oscillation could be seen clearly in cells which had only one small region of closest approach due to an edge-on alignment with the interface. The sequence taken in 0.4 mM NaCl (Fig. 3c, d) illustrates this component of Brownian motion: the colour at greatest separation $d$ is a first-order reddish brown (OPD ~ 500 nm) corresponding to $d = 170$ nm. This distance is less than the calculated value of 230 nm in 0.3 mM NaCl for a secondary minimum energy of 5 kT. The attractive force coefficient in the latter case was found to be $9 \times 10^{-14}$ erg ($9 \times 10^{-21}$ J) (Parsegian & Gingell, 1980), which is about twice the value obtained from hydrocarbon water systems. Haydon & Taylor (1968) obtained a value of $5.6 \times 10^{-14}$ erg for a hydrocarbon film in water. Le Neveu, Rand, Parsegian & Gingell (1977) measured the equilibrium interbilayer repulsive force in hydrated lecithin liquid crystals, using an osmotic pressure method. Equating the electrodynamic
attraction with repulsion at the balance point in the case of zero osmotic pressure gives a value of $2.3 \times 10^{-14}$ erg ($2.3 \times 10^{-21}$ J) for the attractive force coefficient.

Brownian motion perpendicular to the interface would be anticipated for a cell stabilized by secondary minimum adhesion where there is a long-range balance of electrostatic and electrodynamic (van der Waals) forces (Curtis, 1973; Parsegian & Gingell, 1980). The depth of the energy well could in principle be obtained from the amplitude of oscillation, but good quantitative data with monochromatic light would be required.

A second problem in image interpretation stems from the fact that reflections from the far side of the cell combine with those from the oil/water interface to produce an interference pattern, which is superimposed on the main pattern derived from the front of the cell (Fig. 31). This was first discussed in the context of tissue cell images by Izzard & Lochner (1976) and has recently been analysed mathematically by us (Gingell & Todd, 1979). When working with white light it is not advisable to avoid this difficulty by increasing the INA because of colour dependence on INA. However, since the reflections from the far side are focused at a slightly different plane they appear weaker when focusing on the main image. Further, for any finite INA, increasing the specimen thickness damps out the interference extrema; working at INA = 1.20 (corresponding to a solid cone of light of angle $\sim 114^\circ$ in hexadecane), the interference pattern is characteristic of very thick cytoplasm when the mechanical thickness exceeds $1.0-1.5 \mu$m (Gingell, 1980). Consequently, when cells are viewed edge-on we would not expect contributions from the far side of the cell which is 8 $\mu$m away from the interface. This is found to be the case; in 0.4 mM a uniform pale yellow is seen, without any superimposed pattern. The effect of changing INA is shown in Fig. 5. The first- and higher-order bands seen in monochromatic light are damped as the INA changes from 0.69 to 1.2, leaving strong zero-order contacts, as discussed by Gingell & Todd (1979).

While it may be legitimate to discuss adhesion-with-a-gap in dilute salt solutions in terms of long-range physical forces, this is not the only possible interpretation of our experiments. Despite the fact that our theoretical predictions fit the experimental observations reasonably well, it is possible that molecular projections from the cell surface whose area is below the limit of resolution of the microscope ($\sim 0.1 \mu$m) bridge the $\sim 100$-nm aqueous gap and are responsible for anchoring the cells to the interface. A decrease in gap thickness with increasing salt concentration and Brownian motion perpendicular to the interface could both occur in this case. Rather special properties would be required of hypothetical bridging molecules – they should be charged in order to extend in saline yet would almost certainly require uncharged tips to avoid electrostatic image repulsion from the oil/water interface, and, even more improbable, should remain elastic after fixation. While no chemical evidence is available relating to the extent of molecular projection from the red cell membrane, glycophorin is hardly a viable candidate as its fully extended extracellular segment (of $\sim 72$ amino acids) could not exceed 30 nm. Also, as it is so highly charged it is hard to see how it could avoid image repulsion from an adjacent oil interface. We have examined the surface of fixed red cells by scanning electron microscopy and find no evidence of gross projections $\sim 0.1 \mu$m. We checked the possibility that the glutaraldehyde
Fig. 5. Fixed red cells adherent to poly-L-lysine-treated glass. ×10000 approx. A, at INA = 1.2 only the 3 zero-order contacts remain black and higher-order fringe irradiances are attenuated compared with the low INA image (B, INA = 0.69).
fixative might polymerize and thus provide a bridging polymer, by repeating the experiments with cells fixed in formalin, which does not polymerize: the results were indistinguishable from experiments with glutaraldehyde fixation. We therefore feel that while we cannot rule out molecular bridging, the improbable properties required of bridging molecules, together with a reasonable good quantitative correspondence with the theory of long-range forces, lends rather more weight to the long-range force explanation.

The change in cell-surface separation with changing ionic strength is qualitatively in accord with predictions from the theory of long-range repulsive forces: electrostatic repulsion decreases as the concentration rises, allowing the underlying attractive force to pull cells to the interface. The colour change sequence of first-order yellow – yellow/white – pale grey – dark grey – black, which was observed as the salt concentration was raised from 0.4 to 72 mM, corresponds with a smooth decrease in gap from ~ 100 nm to ~ zero. A similar sequence of colour changes (starting at whitish grey) has been observed in the case of *Dictyostelium discoideum* amoebae on glass, when the NaCl concentration was raised from 0.1 to 20 mM (Vince & Gingell, 1979). Far greater consistency was obtained in this system than in the case of red cells interacting with glass. Photometry in monochromatic light (546 nm) at high INA (1:20) showed that cells in Na+ or K+ decreased their separation as the concentration rose from 0.1 to 20 mM. Divalent cations Ca²⁺, Mg²⁺ were more effective in reducing the gap. A clear difference between the curves for Ca²⁺ and Mg²⁺ indicates that ion adsorption at the cell surface reduces the surface potential in addition to Debye shielding. These results with a comparable system complement our demonstration of electrostatic repulsion in the red cell salt concentration experiment. Similar qualitative observations have been made by Preston & King (1978) for amoebae of *Naegleria gruberi* on glass.

An electrostatic explanation for concentration-dependent separation of red cells from an oil/water interface is supported by the observations that cells in 0.4 mM NaCl are reversibly adherent, since they can be removed by diluting the electrolyte (Todd & Gingell, 1980). The same is true of cells which remain single in 10 mM NaCl and do not exhibit the lateral interfacial aggregation which occurs at this and higher concentrations. Long-range force theory predicts that cells stuck in a force balance at a finite separation (secondary minimum of potential energy) would be prone to such loss of adhesion as the repulsive force is progressively increased. Cells stuck to a negatively charged metal electrode were also electrostatically repelled as the surface charge density was increased at a constant electrolyte concentration (Gingell & Fornés, 1976). In contrast, cells initially stuck to a positively charged electrode were not removed when the electrode was made highly negative. The latter condition apparently corresponds to adhesion at the oil-water interface characterized by extensive zero-order black fringes discussed below and indicates a relatively highly adhesive primary minimum (molecular) contact. Unfortunately, due to the high reflectivity of metal and the low reflectivity of the cell surface, interferometry cannot be used to measure separations on metals and so test this conclusion.

At salt concentrations ~ 10 mM NaCl cells ‘snap’ down on to the oil-water interface and apparently make intimate contact with it. The water gap is small or
absent. Photometric measurements of zero-order black regions on these cells are consistent with this interpretation. Extensive close contact requires deformation of either cell or interface, and there is little doubt that the oil/water interface deforms. Contact between red cell and rigid glass in sufficiently concentrated saline, or polylysine-treated glass in dilute saline, produces strictly localized black contacts which do not spread to form the continuous broad ring characteristic of interaction with oil. This shows that the interface deforms when cells contact the oil/saline interface. The interference colours which occasionally remain at the centre are probably derived from a lens of water trapped in the concavity of the cell surface. When viewed in a cuvette with illumination parallel to the interface, shadows cast by the dimples made by partial penetration of the red cells into the oil can be seen. Cells which have entered into this state move rapidly toward each other over a distance of several microns, forming aggregates which cannot be expelled from the interface by diluting the medium with distilled water (Todd & Gingell, 1980). The origin of the lateral motion at several μm/s probably lies in a minimization of overall interfacial curvature.

The observations made on glass using fixed cells also show that cells can adhere in dilute saline with a closest approach ~ 100 nm. In physiological saline, stable zero-order black contacts were formed. Irreproducibility of the experiment in our hands lends some uncertainty to the salt concentration corresponding to the ~ 100-nm separation. On poly-L-lysine-coated glass in distilled water the results were more consistent: all cells adhere with localized zero-order black contacts resulting from maximal electrostatic attraction between the negatively charged cell surface and the positively charged glass. This is completely different from the result in distilled water using untreated glass: under these conditions electrostatic repulsion is maximal and no cells adhered.

Using unfixed cells in isotonic sucrose we observed pale-yellow closest contacts in 0.5 mM-NaCl and similar results were obtained with fixed cells using the same batch of glass. The theoretical effect of sugar on electrodynamic forces, for which experimental evidence is available (Le Neveu et al. 1977) is far too small to be observed in our system. Since sugar is known to have a negligible effect on electrostatic interactions in water (Malmberg & Maryott, 1950) there is justification for considering sucrose inert insofar as modification of long range forces is concerned. This leads to the conclusion that glutaraldehyde-fixed and unfixed cells, despite a small change in surface charge (Vassar et al. 1972; Weiss, Zeigel, Jung & Bross, 1972; Todd & Gingell, 1980), can behave similarly in their adhesive interactions. This result widens the applicability of our results obtained with fixed cells.

In 2.0 mM NaCl, unfixed red cells formed zero-order black contacts. In contrast, unfixed red cells in ~ 0.1% serum protein were more weakly adherent resulting in rolling grey contacts, even in 145 mM NaCl. Such reduction in adhesiveness due to protein adsorption indicates that under physiological conditions the physical attractive forces will be significantly weakened by protein or glycoproteins at interfaces. Adsorption of protein is not, however, so marked as to mask completely the underlying surface, as found by Harris (1973). Pretreatment of glass with poly-L-lysine prevented the formation of rolling grey contacts, giving small stable black adhesions in the presence of 0.1% protein, a mode of contact intermediate between the extremely
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strong contact seen on poly-L-lysine-treated glass and the far weaker contact characteristic of glass with absorbed protein.

The existence of a gap between cells suspended in very dilute saline and a hydrocarbon or glass interface does not imply that living cells in physiological media enjoy a similar mode of adhesion. In physiological saline the Debye length is only 0.8 nm, as opposed to 20 nm in 0.4 mM NaCl. A gap of ~ 100 nm in 0.4 mM saline, representing a secondary minimum adhesion, would collapse to around 7.0 nm on increasing the salt concentration to 145 mM (Parsegian & Gingell, 1973). This is probably of the same order as the external projection of surface protein and glycoprotein from the membrane bilayer: consequently it is unreasonable to discuss cell surfaces interacting at such short distances in terms of smooth planes. Secondary minimum stabilization of cells in physiological saline may therefore be an ill-defined concept and it seems likely that there will be extensive ‘primary’ contact between the macromolecular surface membrane components of contacting cells under physiological conditions. In this limit of close approach both long and short range forces will act in inseparable synergy.

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