STUDIES ON INTERCELLULAR ADHESION

by

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SUMMARY

Dielectric constant measurements were made, in the frequency range 100 kH_3 to 1 MH_3, on the compounds glycine, diglycine, D-sorbitol, dextran (m.w.t. 15-20,000) and Ficoll (m.w t. 400,000) in aqueous solutions of low specific conductivity ($< 200 \times 10^{-4}$ ohm^{-1} m^{-1}) normally buffered to pH 7.45. The values obtained were used to predict the dielectric constant of Hanks-199 tissue culture medium, to which various concentrations of these compounds had been added.

Single-cell dispersions of two primary chick embryonic tissues, 7-day neural retina and 5-day limb bud, were prepared in tissue culture media of varying dielectric constant and also in suitable controls. Ionic strength, pH and osmolarity were kept equal in experimental and control media.

Selected cell dispersions were examined by means of particle electrophoresis in order to determine their electrokinetic potentials. It was found that 7-day neural retina tissue had a mean value for the zeta potential of -13.88 mV $\pm$ 0.04 S.E. in unmodified medium, this value falling slightly in the media of differing composition. The 5-day limb bud tissue gave a value for the zeta potenti of -14.37 mV $\pm$ 0.03 S.E. in normal medium, again showing a slight falling off in the modified media. The results were interpreted as showing that no significant adsorption

of added compounds was occurring onto the cell membranes, thus changing their surface properties.

Cell suspensions in media of a range of dielectric constant were subjected to a laminar flow shear gradient in a Couette viscometer. The effect of this shear gradient was to bring about collisions between the cells and thus to effect aggregation of these suspensions. By measuring the total number of particles in the suspension at timed intervals a parameter was calculated for each aggregation, the collision efficiency. The collision efficiency is a measure of the rate of aggregation and also can be used to calculate the total energy of adhesive interaction of the cells. The collision efficiency was used to calculate this parameter from aggregation studies in media of varying dielectric constant. It was shown firstly that 5-day limb bud tissue gave much lower values for this parameter (termed the force constant) than did 7-day neural retina. In the control media, the former gave a value of 3.66×10^{-26} J. and the latter 5.65×10^{-2}
Secondly it was discovered that in both tissues there was a steady increase in the adhesive interaction of the cells (as measured by the force constant) with increasing dielectric constant of the aggregation medium, the 5-day limb bud being far more sensitive than 7-day neural retina in this respect.

These observations were discussed in relation to the lyophobic colloid stability theory of cell adhesion. The results were found to be consistent with this theory when account was taken of recent theoretical studies into the effect of the dielectric properties of a medium on the attractive energy of particles suspended in that medium.

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INTRODUCTION

Of paramount importance in all investigations concerning intercellular interactions is the role played by the cell surface membrane in these activities. In order to be able to understand the fundamentals of these processes it is first necessary to elucidate something of the structure and behaviour of this limiting membrane. Based upon the ideas of Gorter and Grendel (1925), Danielli and his co-workers Davson and Harvey (1935, 1943) were able, after investigations into the electrical, interfacial and permeability properties of membranes, to account for their results by the use of the following elaborated model. The theory proposed that the membrane structure consists of a sheet of lipid molecules, two molecules in thickness with the polar groups directed outwards and the non-polar hydrocarbon chains inwards and perpendicular to the plane of the membrane. The surfaces ofthis so called "bimolecular lipid leaflet" are coated by an adsorbed layer of protein.

Direct observation of the cell membrane to test this theory only became possible with the development of the electron microscope. Studies on myelin, shown to be derived from the cell surface of Schwann cells (Geren 1954, Robertson 1957) when combined with the results of X-ray diffraction investigations (Finean 1953, Fernandez-Moran and Finean 1957) tended to confirm the Danielli-Davson hypothesis. Similar structures were also discovered in a variety of sub-cellular components, and the structure elucidated for myelin was readily extended to all other

membrane structures by Robertson (1959, 1967) who put foreward the "unit membrane" concept. This postulates that the membranes of all cells possess the same basic structure, in essence that of the Danielli-Davson model, a bimolecular leaflet composed of phospholipids, coated with protein or mucoprotein and/or mucopolysaccharide on both sides. This model has successfully interpreted the pattern of staining observed in the electron microscope, where under suitable conditions, two densely staining lines appear at distances roughly corresponding to the separation of the hydrophilic moieties of two phospholipid molecules arranged in such a bimolecular structure (Robertson 1959).

Recently the "unit membrane" concept has been subject to criticism by several workers (Korn 1966, Lucy 1968, Chapman and Wallach 1968) prompted by the belief that such a highly specialised component of nerves as myelin may not have the typical structure or composition of other cell membranes. Variations in the chemical compositionof membranes have been examined by many workers (de Gier and van Deenen 1961, Ponder 1949, Maddy and Malcolm 1965, Neville 1960, Woodin and Wienekeke 1966) and Elbers (1964) has shown that the overall width of the triple-layered plasmalemma as observed in electron micrographs can vary between 50Å and 130Å (5 to 13 nm) although methods of preparation will influence these separation distances. When this evidence is taken with X-ray diffraction results (Luzzati 1968, Luzzati and Husson 1962) showing stable configurations of phospholipids

in water other than the bimolecular leaflet (a basis for interpretation of micrographs) some workers have felt that the unit membrane hypothesis may be misleading.

Luzzati (1968) has shown from X-ray diffraction studies that a variety of possible structures exist for bulk phospholipids in water, dependant on physico-chemical conditions. He has suggested that the conditions present in the living cell are close to those at which a phase transition may occur from a bimolecular leaflet to a state with hexagonally packed cylindrical micelles, having the ionic groups directed to a core of water. To substantiate the claims of Luzzati, a number of workers have been able to obtain micrographs which seem to indicate the presence of micellar structures in membranes (Sjostrand 1963; Nilsson 1965, Blasie and Worthington, 1968). Lucy (1968) has proposed a detailed model of a membrane constructed from lipid micelles on the basis of observations of lipid complexes using a negative staining technique (Lucy and Glauert 1964). The membrane may be assembled from sub-units in a hexagonal array with a layer of protein or glycoprotein on either side of the micellar components. This model allows for random movements of the micelles, with pores appearing between some of the sub-units and with the possibility of phase transitions occurring to the bimolecular leaflet configuration.

However at this stage sufficient data required to critically test any particular model of the cell membrane is not yet available. It may prove to be that differences

in membrane properties may result from the presence of specific peptides proteins or glyco materials rather than any fundamental structural variations of the bi-molecular leaflet model.

At the present moment then relatively little is known of the structural organisation of the cell membrane though research in this field is advancing. Similarly investigations into the ways in which these membranes interact has produced as yet only scant information as to the processes involved. This is chiefly due to the extremely difficult technical problems associated with investigations into the behaviour of membrane structures. Loevenstein (1967) has shown the importance of interactions of membranes with one another and Rubin (1966) has discussed the relationships between carcinogenesis and the cell surface. One field in which membrane interactions are though to be of importance is that of cell adhesion which has been implicated in phenomena such as cell division metastasis and morphogenesis (Curtis 1962).

The oldest of the theories concerning cellular adhesion is the cementing theory, in which cells are thought to be attached to each other by a cementing material present in the gap between the cells. An extension of this theory proposes the existance of specific cements responsible for the adhesion of given cell types (Moscona 1961, 1962). Evidence cited for or against this hypothesis is equivocal, the action of general enzymes such as trypsin which is much used in dispersing cells is said to suggest

the presence of a cementing substance attached to cells by covalent bonds. However, it is equally possible that enzymic action may cause changes in the surface properties of the cell, also the enzyme may attack tissue such as collagen which has primarily a strengthening role. More recently a modification of the cementing theory has been proposed by Steinberg (1958, 1964) and Pethica (1961). It is thought that calcium ions, suggested to be of great importance in cell adhesion, can form ion-pairs or triplets with carboxyl groups on the cell surface (Haydon and Seaman 1962) thus acting as a bridge between the cells. One important aspect of this theory is that the cells are considered to be in molecular contact with a separation of less than 2nm (20Å) between the two apposing surfaces. This view has been supported by the work of Wilkins (1962) which suggested that leucocytes flocculated at zero point of charge were adhering with molecular contact. Curtis (1967) in a review of the subject of cell adhesion has pointed to a considerable body of evidence for the existence of two major classes of cell adhesion. The first is an adhesion with separations of less then 2nm (20Å), the cells being in molecular contact and not easily dispersed. Although certain specific cell to cell adhesions such as sperm to egg may be of this type, it appears that many cells show an adhesion of a second type. In this case a separation of 10 to 20 nm (100 to 200Å) exists between the membranes. This is a relatively weak

adhesion as compared with the first class and is sensitive to ionic conditions of the media, particularly with respect to divalent cations. In this light the specific mechanism theory seems less viable than previously supposed as the effect of calcium ions can be explained on other lines rather than on the formation of bridges. Also it is evident that the theory cannot accommodate the weak adhesion with the 10 to 20 nm gap without invoking the possibility that calcium ions may form bridges between the cement materials supposed to be covering the cells.

From a theory proposed by Derjaguin and Landau (1941) and Verwey and Overbeek (1948) on the stability of lyophobic colloids, Curtis (1966) has suggested that cell adhesion is the result of the balance between opposing physico-chemical forces. In the majority of cases, adhesion is the result of a weak reversible flocculation with the cell surfaces separated by a gap of some 10 to 20 nm by a balance between attractive and repulsive forces. The theory also allows for adhesion at close separations of less than 2 nm with the possibility of molecular contact. According to this theory the repulsive force is a consequence of the presence of charged groups of the same sign on two opposing cell surfaces, giving rise to an electrostatic force of repulsion on account of the surface potential. The attractive force is the London-van der Waals force. The London dispersion force is determined by the nature of the adhering particles and of the material present in the gap between them.

The magnitude of the two forces falls off with distance, but attractive and repulsive forces obey different laws. The London attractive force exhibits an inverse power law while the electrostatic repulsive force falls according to an exponential law. It is predicted that when these two forces interact, two distinct adhesions at different separations can occur when the net adhesive and repulsive energies are balanced. The first is a close adhesion when the adhesive forces are maximal, with a separation of only a few nanometers, this is termed an adhesion in the primary minimum. The second adhesion is a weak reversible interaction with a separation of 10 to 20 nm and is termed an adhesion in the secondary minimum. Whilst no appreciable barrier exists to prevent the approach of distant particles (cells) to the secondary minimum, the nature of the repulsive force is such that a large potential energy barrier generally prevents the particles from adhering in the primary minimum. The idea of an adhesion occurring in the secondary minimum has been criticised on occasion but Schenkel and Kitchener (1961) have provided good evidence that polystyrene particles so adhere. Also Curtis (1967) has pointed out that physiological conditions would favour a weak adhesion in the secondary minimum with a separation varying from 8 nm to 11.5 nm depending on the values chosen for surface potential and London force constant. Thus this theory predicts adhesions which closely correspond to the two classes of adhesions discussed for cells above. Furthermore the ionic relations

of the adhesion of tissue cells are correctly explained by this theory. High ionic strengths would be expected to reduce the surface potential and as a result reduce the electrostatic force of repulsion - this promoting adhesion in the secondary minimum. Divalent cations are more effective in reducing the surface potential than are monovalent cations, hence the importance of calcium ions in cell adhesion. The theory also predicts the relative non-specificity of adhesion, where cells will adhere to a wide range of other cell types and other surfaces such as glass normally alien to them. At present it seems that the lyophobic colloid approach of Curtis (1966) provides a more satisfactory explanation for cell adhesion than the other theories outlined above, though to date the critical experimental data required to properly evaluate the various theories does not yet exist.

There is even less data on the role of the cell membrane constituents in the adhesion of cells. There has been speculation about the action of specific molecules thought to be present on the lipid membrane (Weiss,L.1960) particularly about the significance of the carboxy groups of compounds such as sialic acid (Cook et al 1961). More recently mechanisms involving the complex carbohydrate present on the surface have been proposed by Roseman (1970). These theories are based upon the discovery by Oppenheimer et al (1969) that the conversion of non-adhesive to adhesive teratoma cells appears to require the synthesis of L-glutamine, it being thought probable

that adhesive substances are formed by metabolic reactions in which L-glutamine is utilised. One theory predicts interactions between carbohydrates of two apposing cell membranes utilizing the formation of hydrogen bonds between the glycose units. A second proposal by Roseman (1970) is of an enzyme-substrate interaction. Cell surfaces are supposed to contain both substrate and enzyme and the binding of one to the other results in a stable adhesion. Results of investigations into these theories are very preliminary as yet (Roth McGuire and Roseman in prep.).

A major restriction to the investigation of the forces which are involved in cellular adhesion has been the lack of an adequate technique allowing an absolute measurement of cell adhesiveness to be made. Previously measurements have been made of the force required to separate cells (Brooks et al 1967) but these methods can be criticised on a number of grounds; that intercellular material such as collagen may contribute to the resistance of cells to dispersion, or that the force measured may be that required to cause rupture of the cells. Because of these problems, attempts have been made to measure adhesiveness during the formation of cellular aggregates. Moscona (1961) used the size of cell aggregates, formed when a suspension of single cells was shaken, as a measure of adhesiveness, though it is difficult to derive an absolute measurement from such results. Curtis (1969) has described a technique

which allows such a measurement to be made from the rate of aggregation of cell suspensions. This technique is based on two fundamental concepts. Firstly, movements of the medium in which cells are suspended tend to bring the particles together and then re-separate them as the particle is passed by another particle having a relatively greater velocity. If the adhesive force is sufficient to bring the particles close enough together a stable adhesion will form to resist the break up of this aggregate. The second concept is that the proportion of collisions resulting in adhesions is a measure of the adhesiveness. This proportion, termed the stability ratio or collision efficiency, has been measured previously though rarely directly. Although Fuchs (1934) derived an absolute measure of the adhesive energy between particles from measurements of the stability ratio, his treatment is not applicable to suspensions of cells subjected to hydrodynamic forces, since it treats the particles as acting under the action of Brownian motion. Curtis and Hocking (1970) have developed a new technique for deriving the adhesive force from the collision efficiency which is applicable to measurements made in suspensions subject to a laminar shear gradient. This technique has been used in the present study of the flocculation of single cell suspensions and will be described fully below (see Materials and Methods).

One experimental approach which may provide more evidence with which to evaluate the various theories of cell adhesion is that concerned with the physical parameter termed the dielectric constant. This function may be considered as the charge dissipating quality of the medium and is defined by Coulombs equation. In a consideration of the possible changes in the adhesive properties of cells with dielectric constant, the lyophobic colloid stability theory proposed by Derjaguin Landau: Verwey and Overbeek indicates that the variation of potential and force with distance is dependant upon the dielectric constant of the medium between the particles (Kruyt 1952). Theoretical calculations of the total interaction potential energy curves for particles in which the dielectric constant of the medium has been varied show that adhesiveness can be affected by this parameter (Brooks et al. 1967, Gingell 1971). On the other hand, the dielectric constant value of the suspension media is not a factor to be considered in the other theories of cell adhesion outlined earlier, since these generally depend upon specific chemical interactions between cell surfaces rather than on bulk properties of the medium. Experimental evidence concerning the adhesive properties of cells over a range of dielectric constant values should therefore be of some help in elucidating which if any of the current theories of intercellular adhesion is the more relevant.

Changes in the dielectric constant of the medium affects both the energy of repulsion and the energy of attraction. The electrostatic value of the dielectric constant of the medium must be used in order to determine the effect on repulsive energies. The dielectric constant for various phases of biological systems has often been assumed to be similar to that of water, but Pollack et al. (1965) have pointed out that the dielectric constant is dependant upon the macromolecules present in solution. This group suggests that as the electrostatic dielectric constant of the media is increased, there is an overall decrease in the potential energy barrier between two charged particles, though in this study attractive energies were not treated. However, Brooks et al. (1967) and Gingell (1971) have shown that for a given value of the attractive energy, and increase in dielectric constant of the intercellular gap will increase the repulsive term with a consequent increase in the potential energy barrier and a decrease in the magnitude of the secondary minimum. The range of possible stability in the secondary minimum is shifted to higher values of the London-Hamaker constant.

While satisfactory theoretical treatments of the repulsive electrostatic energies have been developed, previous investigations of particle interactions have been limited by lack of a suitable treatment of the London-van der Waals attractive energy. Until quite recently, measurements of van der Waals forces were indirect being mostly based on the force required to

break adhesions between surfaces. Derjaguin and Abricossova (1954) were among the first to publish direct measurements of the forces between surfaces as a function of separation, the smallest distance of approach being about 100nm. Theoretical estimates of energies have, however, been limited by several ad hoc assumptions, the most important of which are pairwise additivity of individual atomic interactions and an arbitrary "dielectric factor" correction for any intermediate substance between interacting particles. A completely different approach which uses the bulk dielectric properties of the material has been developed by Lifshitz (1956). This theory circumvents the assumptions of older theories because it includes all the many body forces, retains the contributions from all interaction frequencies and deals correctly with the effect of intermediate substances (Dzyaloshinskii, Lifshitz and Pitaevskii 1960). The theory of Lifshitz has been successfully utilised by Tabor and Winterton (1968) in a direct measurement of the van der Waals forces between mica sheets. The method is thought to have full generality and is applicable to any body, it also automatically takes into account retardation effects which become important for sufficiently large separations between the bodies (Lifshitz 1956). In the later paper (1960) where the theory is extended to cases where the gap between the bodies is filled by some medium it is shown as a general conclusion that any two identical bodies will always attract one another whatever

the nature of the intervening layer, and that the greater the difference between the dielectric properties of the two identical particles and that intervening medium, the greater the attractive force between the particles. Applications of the Lifshitz theory to calculations of van der Waals forces across thin lipid films (Parsegian and Ninham 1969) and in lipid-water systems (Ninham and Parsegian 1970) have shown that while it is necessary in principle to use total macroscopic dielectric data from the component substances for a full analysis, in practise it is possible to use only partial information to perform satisfactory calculations if this exists.

Because of the growing amount of knowledge becoming available in the literature concerning theoretical aspects of the dielectric constant in relation to particle interaction, it was thought appropriate to relate this knowledge to the problem of cell adhesion. This thesis describes some preliminary work on this problem.

1. Determination of Cell Adhesiveness - theory

In a suspension of particles, collisions can be brought about either by Brownian motion or by movements of the medium. In the latter case, if the movements are such that the particles are brought into collison by a laminar shear flow of rate G, it is possible to derive a theoretical frequency for collision between particles. Von Smoluchowski (1916) showed that for particles of radius ri and rj, at concentrations ni and nj, the total number of collisions per unit time interval, bij, during a flocculation in a laminar shear gradient is given by the relationship

$$bij = {}^{4}/_{3}\ G\ ni\ nj\ (ri + rj)^3 \qquad (1)$$

where G has the dimensions of reciprocal time. This equation cannot be applied to the whole course of a flocculation because once an appreciable quantity of two-particle aggregates have formed, these may collide to give three or four bodied aggregates. Swift and Friedlander (1964) have developed a particle size distribution by integrating the total number of collisions for all classes of aggregate size. They were able to demonstrate that this size distribution function is mathematically consistent with the Von Smoluchowski theory of flocculation and that the total number of particles at time t, $N_\infty t$, compared with those

at the start of flocculation $N_{\infty o}$ is given by

$$\ln \frac{N_{\infty t}}{N_{\infty o}} = \frac{4G\phi t}{\pi} \qquad (2)$$

where G is again the shear rate and ϕ is the volume fraction of particles in the suspension. It has been pointed out however (Fair and Gemmell 1964) that the Smoluchowski equations for orthokinetic flocculation i.e. under conditions of shear could not be solved analytically.

Movements of the medium will tend to re-separate the particles and only a proportion of all collisions may be effective in producing aggregates. The probability that a collision between two particles results in an adhesion is termed the collision efficiency (stability ratio) and equation (2) becomes

$$\ln \frac{N_{\infty t}}{N_{\infty o}} = -\frac{4G\phi\alpha t}{\pi} \qquad (3)$$

where α is the collision efficiency. The collision of small particles, in that the Reynolds number for their motion is less than unity, is resisted by the hydrodynamic force of translation and rotation of the particles, which is inversely proportional to the gap between the surfaces (Brenner 1961). In the absence of an adhesive force, the particles would be brought together by the shear gradient and then rotate around each other to re-separate, and no doublets would be formed.

An adhesive force which could counteract the hydrodynamic force, and thus allow an adhesion to occur during a collision of two particles, is the London-van der Waals force. The London dispersion force is an attractive force to which all molecules are subject. It has been proposed as the force responsible for the coagulation of lyophobic colloids, as it is the only attractive force of sufficient generality (Overbeek 1952). Using a correspondence model, the London force can be pictured as due to the rapidly fluctuating dipole moment generated by the zero point energy of the dispersion electron on an atom. The frequency of this fluctuation is of the order of 10^{15} to 10^{16} per second which is comparable to the frequency of electronic movements (Overbeek 1952). The electromagnetic oscillations set up by this fluctuating dipole will polarise the dipoles of any neighbouring atom as the coupling of two such electrical oscillators results in a gain in energy (Moelwyn-Hughes 1961). This results in an attractive energy of interaction between two like molecules. The importance of the London-van der Waals force lies in the fact that the London energy is approximately additive and a much larger attractive force exists between conglomerations of atoms, hence the significance of this force in the study of colloid flocculation.

For two parallel plates, of a thickness large in comparison to the distance between them, the attractive

energy Va can be given by

$$Va = \frac{A}{-48\pi d^2} \qquad (4)$$

where A is the London Hamaker constant for the material of the plates and d is the half-distance between them (Overbeek 1952). The summation of the London energies involved in the interaction of two spherical particles yields

$$Va = \frac{-Aa}{12H} \qquad (5)$$

where a is the particle radius and H the closest separation of the particles.

Experimental evidence for the existence of this force has been provided by Derjaguin et al (1954), Overbeek and Sparnaay (1954), Schenkel and Kitchener (1961) and Tabor and Winterton (1969). As the energy of London forces at small separations is inversely proportional to the square of the gap, when a collision occurs between two particles they may be able to overcome the hydrodynamic repulsion which is inversely proportional to the gap.

Curtis and Hocking (1970) have analysed the hydrodynamic forces acting during the collision of two particles and the influence of the London dispersion force on such collisions and hence upon the value of the collision

efficiency. Taking the case of two equal spherical and electrically neutral particles, so that no electrostatic repulsive force exists due to the interaction of electrical double layers, they derive equations for the motion of the sphere which is governed by three forces - (i) the effect of the shearing motion on the particles (ii) the hydrodynamic forces (iii) the London force of attraction. These lead to a relationship for the London-Hamaker constant which is based on the ratio of the London force to the Stokes force and is given by

$$A = 72\pi \, na^3GK \qquad (6)$$

where n is the viscosity of the medium K is an interaction parameter which depends, because of retardation, on the wavelength λ of the intrinsic electronic oscillations of the atoms. Curtis and Hocking have calculated values for the collision efficiency as a function of the parameter K for different values of λ. Thus it is possible to estimate an experimental value for the collision efficiency, derived from measurements of orthokinetic flocculation, from this to evaluate K and hence from a knowledge of particle geometry to work out the London Hamaker constant for the retarded force, and the attractive energy for particles in a suspension.

This method which was confirmed for the flocculation of polystyrene beads in aqueous suspensions, can be

applied to adhesions in the primary minimum where no appreciable electrostatic repulsion exists to provide a potential energy barrier to the approach of the particles; or to those in the secondary minimum though there are at present computational problems in this case. The treatment of Fuchs (1934) is applicable where a potential energy barrier exists. The treatment of Curtis and Hocking shows that collision efficiency is dependent on the shear rate (equation 6) whereas Fuchs' treatment has as its rate determing step the frequency with which Brownian motion energy exceeds a certain value, allowing the potential energy barrier to be surmounted. This is not dependant on shear rate and the collision efficiency will thus be independant of shear rate. This provides a method for assessing the applicability of the two treatments. It should even be possible to modify Fuchs treatment so that flocculations in a shear gradient with a potential energy barrier may be dealt with.

2. Determination of Cell Adhesiveness - experimental

The relationship given by Swift and Friedlander (1964) allows the collision efficiency for the flocculation of a monodisperse suspension of particles subject to laminar shear flow to be determined from counts made of the total number of particles at timed intervals. The Couette viscometer provides an apparatus in which laminar flow conditions may be achieved with

known stable shear rates, if the instrument is constructed to the principles given by van Wazer et al. (1963). The Couette viscometer consists basically of two concentric cylinders, one suspended freely inside the other with a narrow gap between them. A suspension of particles is placed in this gap and one of the cylinders (the outer) is rotated. Laminar shear flow is established, with a shear gradient across the gap, and the shear rate, G sec^{-1}, is given by

$$G = W \frac{(Ro^2 + Ri^2)}{Ro^2 - Ri^2)} \qquad (7)$$

where W is the angular velocity of the rotating cylinder, and Ro and Ri are the radii of the outer and inner cylinders respectively.

Fairly low rates of shear are necessary so as to allow a number of measurements to be made. Albers and Overbeek (1960) showed that the velocity of flow difference across an aggregate increases with the square of the radius of the aggregate and will tend to break them up. Thus for any given value of collision efficiency, at a given shear rate there will be an equilibrium size for aggregates at which the rate of aggregation is equal to the rate of disruption of the aggregates. The higher the shear rate, the smaller will be the maximum size of the aggregates, and the sooner will the equilibrium conditions be attained. Since the kinetics of the flocculation depart significantly from those given by

equation (3) as the equilibrium condition is reached, it is important that measurements are made before this point. A knowledge of the shear rate also allows an evaluation of the contribution by Brownian motion in effecting collisions, a significant contribution would also result in a divergence from the kinetics of equation (3). Tuorila (1927) derived a relationship for the ratio of collisions produced by shear to those produced by Brownian motion, J/I, given by

$$J/I = \frac{n\,(ri + rj)^3\,G}{2kT} \qquad (8)$$

where kT has the usual meaning. For shear rates as low as 1 sec^{-1} and with particles of radius 5um. at a temperature of 293°K, less than 1% of the collisions will be brought about by Brownian motion. It is therefore possible to achieve aggregations which follow the kinetics of equation (3) by suitable choice of the dimensions and angular velocity of a Couette viscometer.

The Couette viscometers used were designed by I. Cameron Esq. of the Department of Engineering, University of Glasgow and constructed by their workshop. The cylinders were made of EN58B stainless steel, which is resistant to corrosion by saline solutions, and machined to a finish of 10um. The inner cylinder is freely suspended on air bearings while the outer cylinder is rotated to give a constant rate of shear by means of an integrating motor (Ether Ltd. Stevenage, Herts) which

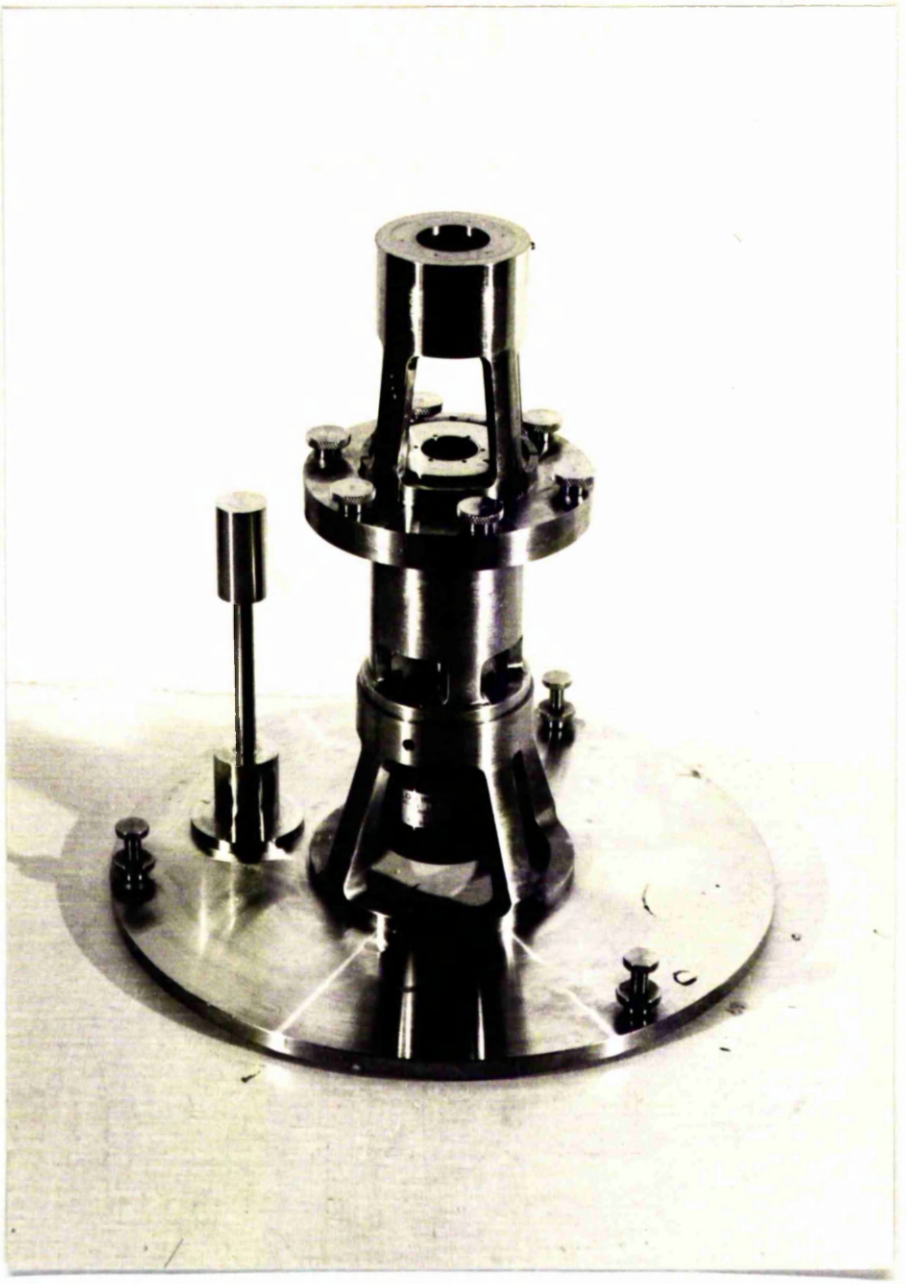


Fig. 1. The Couette Viscometer

Inner bob shown on the left.

runs at a very stable speed. Speed of rotation of the outer cylinder can be varied by changes of voltage or gearbox so that shear rates from 2 sec^{-1} to well above 20 sec^{-1} are easily attainable.

The viscometers were placed in a constant temperature room some hours before use to allow them to reach the temperature at which measurements were to be made. The constructions were aligned vertically by levelling, the construction of the viscometer ensures a correct centering of the inner cylinder inside the outer rotating cylinder, allowing stable shear conditions to be set up once the outer cylinder is rotating. The viscometer is constructed with air bearings pressure being provided by a small air pump during the course of the experiments. (Fig. 1).

3. Measurement of Dielectric Constant - theory

Under ideal conditions, the dielectric constant, ϵ', of a material under test will be proportional to the measured capacitance, Cm, obtained from the apparatus to be described below. However, in practice the ideal is never obtainable and several complicating factors have to be accounted for. Consider a conducting solution between two electrodes of simple geometric form, such as two plane parallel plates. Two problems must be considered, (a) the measurement of the capacitance in such a dielectric cell; (b) the evaluation of the dielectric constant, ϵ', from the measured capacitance, Cm.

The cell may be characterised by its admittance vector composed by a capacitance and a parallel conductance constribution, Cm. The phase angle between this vector and the real axis of the Argand diagram is defined by (Mandel 1965)

$$\operatorname{tg} \varphi = \frac{2\pi Cmf}{Gm} \qquad (9)$$

where f is the frequency of the applied alternating voltage. Neglecting for the moment all contributions to the measured impedence other than those arising from the solution itself equation (9) may be transformed into

$$\operatorname{tg} \varphi = \left(\frac{\epsilon f}{\sigma}\right) \times 5.65 \times 10^{-13} \qquad (10)$$

where σ stands for specific conductivity of the solution. It may be seen that when σ is large and f is small, tg φ attains very small values indeed. This means that the capacitance contribution to the admittance vector is nearly negligible with respect to the conductance Cm. Therefore special impedence or admittance measuring devices will become necessary for the determination of Cm, this is particularly true for solutions of high specific conductance, such as physiological media, at frequencies below 1 MH_3 where ϵ' is less than 100. At frequencies higher than 1 MH_3 the difficulties are eliminated, but are replaced by more conventional ones which are by no means

diminished by the existence of an important conductance contribution.

The second problem to be dealt with is the computation of the dielectric constant ϵ' from the measured capacitance. For each measuring cell one must take into account a residual capacitance Co, which under many circumstances is not a constant but may depend on many factors e.g. the measuring frequency, and a self inductance Lc which is due partially to the cells' connecting devices. Futhermore for conducting media a frequency dependant polarisation due to the electrical double layer at the electrodes will occur. If a metallic electrode is immersed in a solution, a boundary potential is found to exist between electrode and fluid. If an alternating current is passed through the fluid from the electrode, the polarisation potential becomes modulated with an alternating potential. This modulation potential is observed to be proportional to the alternating current density (Schwan 1963) provided that the current density is kept sufficiently small. The polarisation of dielectric cell electrodes is an important source of error in the determination of the dielectric properties of dilute electrolyte solutions. This phenomenon becomes increasingly important as the frequency is lowered and the conductivity of the solution is raised. An analysis of this situation is given by Schwan (1963) and Mandel (1965). In the treatment of Schwan, it is shown that the total capacitance Cm predominantly reflects either the true sample capacitance

Cs at high frequencies or electrode polarisation at lower frequencies. Mandel has given an equivalent circuit for the measuring cell, which has been utilised by Young and Grant (1968), and accounts for the correction factors such as electrode polarisation and lead inductances (Fig. 3). If the total capacitance Cm and conductance Gm are measured then the following relationships hold, as long as $Rc << \bar{G}^{-1}$ (generally true) and when Lc is sufficiently small and frequencies so low (below 1 MH_3) such that $(2\pi fC_sG_s^{-1})^2 << 1$

$$Gm^{-1} = Gs^{-1} + \frac{Ge^{-1}}{1 + (2\pi fCeGe^{-1})^2} \qquad (11)$$

and

$$Cm = Co + \frac{CsGm^2}{Gs^2} + \frac{Gm^2Ce}{1 + (2\pi fCeGe^{-1})^2Ge^2} - LcGm^2 \qquad (12)$$

the parameter Cs is the quantity proportional to the dielectric constant. At high frequencies when electrode impedence does not contribute, equations (11) and (12) simplify to

$$Gm = Gs \qquad (13)$$

$$Cm = Cs + Co - LcGm^2 \qquad (14)$$

However although it is commonly stated that electrode polarisation is negligible above frequencies of 100KH_3

Fig. 2. Guard ring arrangement

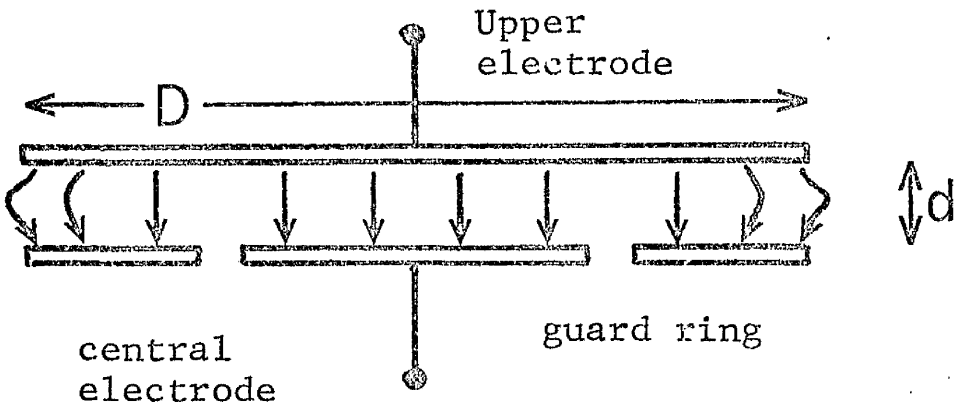
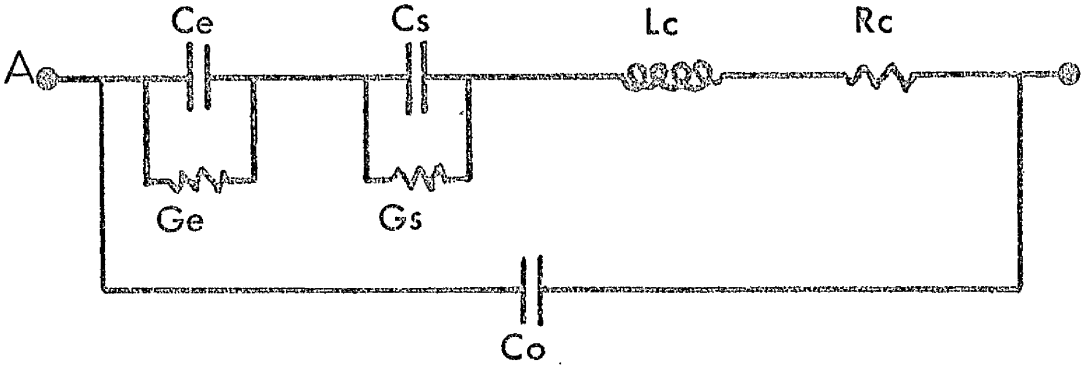
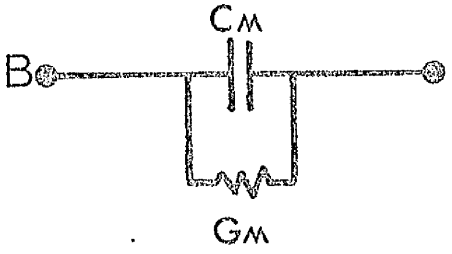


Fig. 3. Equivalent circuit for Cm and Gm

A. Complete circuit for dielectric cell with conducting solution.





B. Circuit as seen by measuring bridge.

Co - residual capacitance
Ce and Ge - electrode effects
Cs and Gs - conducting solution
Cm and Gm - measured values
Lc - inductance
Rc - resistance
$\overline{G}$ - Ge + Gs

(Young and Grant 1968, Schwan 1963), electrode effects may perturb the measurements up to relatively high frequencies. This depends on the nature of the electrode material, its surface, the exact nature and conductivity of the solution and other factors as yet not so well established.

Apart from electrode polarisation a second source of error due to electrode effects is that termed "strayfield". Because electrode polarisation makes impedence measurements on biological systems difficult to carry out, it is often necessary to operate with large electrode distances in order to increase the resistance. This may be seen most readily from the expression

$$Cm = Cs + 1/W^2R^2Ce \qquad (15)$$

where W is the anglular frequency. However, in this case, uncontrolled stray fields may lead to errors in measuring the capacitance. The equivalent electrical circuit diagram (Schwan 1963) consists of a series of capacitors shunted across various sections of the entire sample. A frequency dependence of the apparent properties results while the properties of the sample may not be frequency dependant at all. This effect has been recognised by Jones and Bollinger (1931) and treated in detail by Schwan and Maczuk (1960). Adequate control of stray field components is obvious. The most straight-forward method for the control or elimination of stray

fields is by the use of "guard ring" electrodes, fig (2) shows the application of the principle. In this technique the potential of the guard ring is made identical with that of the central electrode. In such cases the impedence of the sample under the central electrode will be determined directly uninfluenced by stray field problems, since the field configuration is kept perpendicular to the central electrode. A guard ring arrangement operates satisfactorily in establishing a uniform sample field only if the total diameter of the arrangement D is large by comparison with the electrode separation d. This requirement particularly at lower frequencies will lead to great difficulties with electrode polarisation. It can be seen that in measurements of capacitance in a dielectric cell a compromise must be made to minimise the two major electrode effects. A suitable choice of apparatus, frequency and method of measurement and operating distance of the electrodes must be dependant on the particular measuring problem involved and the accuracy of the final result desired.

Several methods have been described to calculate the sample capacitance and dielectric constant from the total measured impedence. A brief review of the techniques attempted in this investigation follow together with a more detailed account of the final method of choice.

(1) Linear extrapolation of Cm to infinite frequency (Oncly 1938):- In this method Oncly was able to plot graphs of Cm against log f and found that at high frequencies Cm tends to Cs. This method requires that rather a large frequency range be covered (25 kH_3 to 2.5 MH_3) and that the solution under test does not present a dielectric relaxation over this frequency range (Ferry and Oncly 1941). A frequency dependant power function is used as a correction for polarisation which is given as $f^{-3/2}$ for platinum electrodes, but the value for stainless steel is uncertain.

(2) Linear plot of Cm against Gm^2 (Young 1967):- Where electrode polarisation is negligible, equations (13) and (14) are valid. Assuming that Co and Lc are constant for any one solution, a straight line graph will be obtained by plotting Cm against Gm^2, Lc being the slope of the line. The variation of Co and Lc with Cs can be found by making measurements on solutions of known dielectric constant. The method is useful and was used to a small extent in the estimation of inductance (Schwan 1963). However, the possibility of disturbing electrode polarisation effects cannot be ignored even at kH_3 frequencies using stainless steel electrodes.

(3) Extreme electrode separations (Schwan and Maczuk 1960): Appropriate use of the pertinent equations given shows that a logarithmic plot of the apparent dielectric

constant as a function of electrode separation will enable the true dielectric constant to be estimated. Extreme values of distance of electrode separation are chosen to emphasise independantly either polarisation contributions (short distance) or stray field ones (long distance) and when these terms are subtracted from the experimental data one obtains the correct sample dielectric constant. In practice very large interelectrode separations are required, in the order of 100 to 200 mms, to ensure that only stray field effects are measured at large distances.

(4) Small electrode separation variation (Fricke and Curtis 1937):- In this method measurements of capacitance are made for two slightly different spacings of the cell electrodes. When this is done, the dielectric constant of the solution can be obtained without knowing the absolute value of the polarisation impedence, the latter appears only as a parameter in the calculations and is eliminated. For two different electrode positions the following equations apply (Schwan 1963)

$$Z_1(S) + Z(P) = Z_1(0) \qquad (16)$$

$$Z_2(S) + Z(P) = Z_2(0) \qquad (17)$$

from which by subtraction

$$Z_1(S) - Z_2(S) = Z_1(0) - Z_2(0) \qquad (18)$$

where Z is impedence, S is sample, P is polarisation, O is observation.

It can be seen that equation (18) is independant of electrode polarisation impedence. The technique is rigorous and accurate but is based on a certain number of assumptions; (a) Constancy of polarisation impedence is necessary as the electrode spacing is varied (b) $Gs = \bar{G}$, this will depend on the importance of electrode conductance (c) Co must be a constant as a function of distance, d (d) both Cs and Gs must be proportional to reciprocal distance d^{-1}. Assumptions (c) and (d) are only verified in an ideal cell but all the assumptions are generally fulfilled and in practice can be checked. The limitations of the technique are that polarisation impedences become large in comparison with sample impedences at low frequencies and that minor errors in the determining of observed impedence reflects in large relative errors of sample impedence. This technique provides a simple straightforward and accurate measure of the dielectric constant. This is calculated from the measured capacitances C_1 and C_2, corresponding to the electrode spacings d_1 and d_2 by means of the equation (Shaw 1942)

$$\epsilon' = \left[d_2^2(C_2-Co)-d_1^2(C_1-Co)\right] / K(d_2-d_1) \qquad (19)$$

where Co and K are constants for a particular dielectric cell.

This method, with its ability to correct simply for electrode effects (Shaw 1942) was finally chosen, after a study of all the techniques mentioned above, as the most suitable for the present study.

4. Measurement of Dielectric Constant - experimental

In order to avoid major contributions from electrode polarisation, measurements of capacitance and conductance were determined in the low radio frequency range. The apparatus consisted of a signal generator, bridge, detector and measuring cell. The signal generator and detector was type SR268 manufactured by the Wayne Kerr Co. Ltd.,New Malden, Surrey, and is a general purpose bridge source and detector covering the frequency range 100 kH_3 to 100 MH_3. Push button attenuators are provided to control the source output level and detector input sensitivity. A meter facilitates visual detection of the null point. The accuracy of frequency calibration is claimed to be $\pm$ 2% of the indicated value, and at each frequency used the source was checked using a computing counter (Racal 9521,Racal Instruments Ltd., Windsor, Berks). The bridge was a radio-frequency type B201 also manufactured by the Wayne Kerr Co. The B201 is a three-terminal, transformer ratio-arm admittance bridge providing measurements of capacitance and conductance over the frequency range 100 kH_3 to 5 MH_3. The main advantages of the transformer bridge relevant to this investigation lies in its ability to measure

very small capacitance values, any strays present in the bridge can be balanced out for the chosen frequency before measurements are made, also any spurious impedences connected to the unknown terminals can be completely counteracted by the appropriate use of the third terminal of the bridge. The overall bridge accuracy is given as ± 1% for the measurement ranges 0.001 pF to 0.1 uF for capacitance and 1 u Ω^{-1} to 1 Ω^{-1} for conductance. The measuring cell was a micrometer Permittivity jig D 321 manufactured by Wayne Kerr, this was initially designed for measuring the dielectric properties of solid materials, but for the present work it was modified by gluing a piece of Perspex 25 mm deep with a 52 mm diameter hole in it, centrally onto the base plate so that liquids could be placed between the electrodes. The jig is a 3-terminal device with a guard ring design to ensure that the field through the sample was uniform, the micrometer is fixed to the movable upper electrode and marked off in units of 0.0025 mm. All connections between units were made with grounded coaxial cable with lead lengths being kept to an absolute minimum, this minimised sources of error due to large inductance values and easily enabled stray capacitances to be balanced out. The whole assembly is shown in fig (4).

All dielectric constant determinations were made at the same temperature at which cell aggregation and electrophoresis were conducted, the whole apparatus being set up in a constant temperature room. In all the

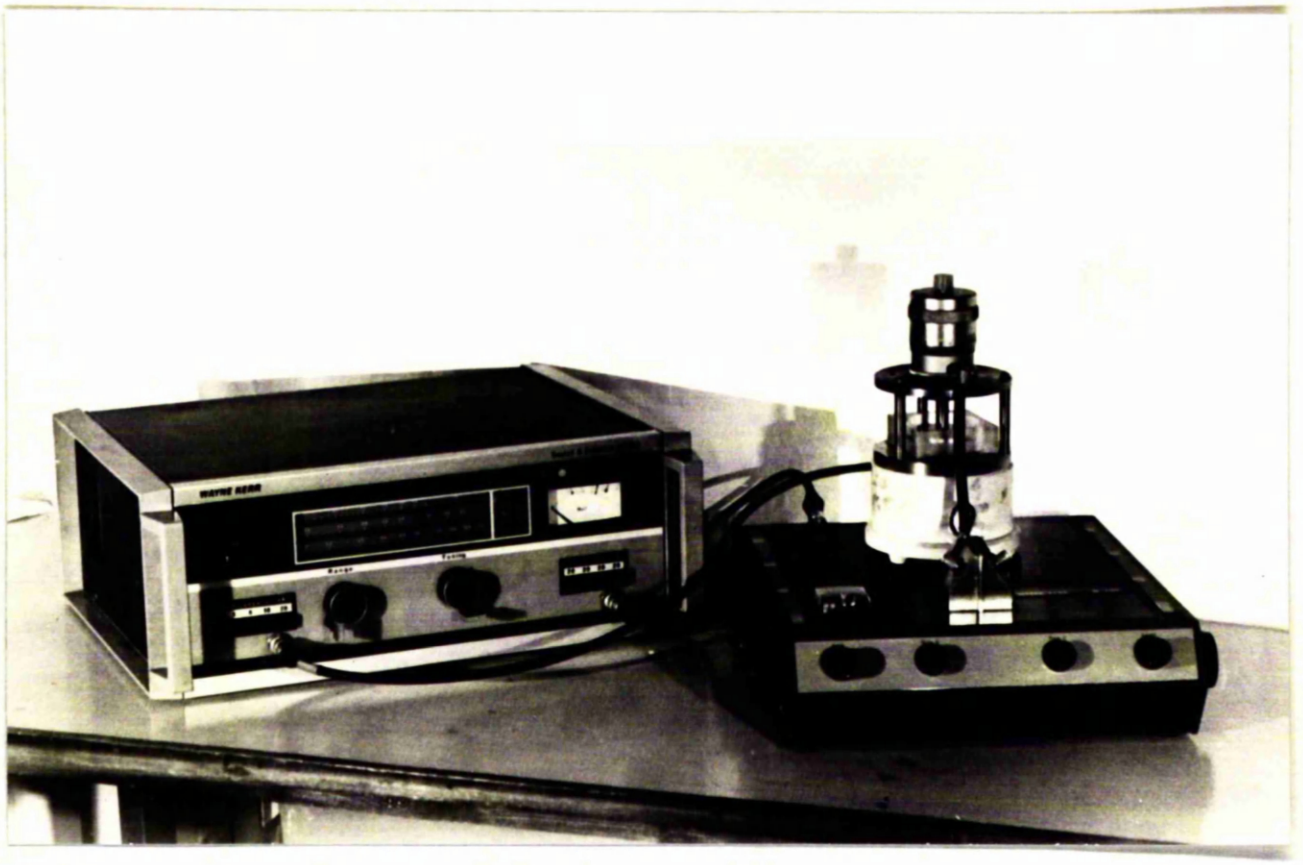


Fig. 4. Dielectric measurement apparatus

showing: Source and Detector
Measuring bridge
Dielectric cell

results given here the method of Shaw (1942) was used except in the determination of inductance (see Results). In this method the bridge was first balanced at the chosen frequency with connecting leads attached, the jig was then assembled, solution introduced between the electrodes and the measured capacitance and conductance were determined at two electrode spacings. The procedure was then immediately repeated to check the reliability of the first set of values. Measurements could be made on solutions with specific conductivities as high as 200 x 10^{-4} ohm^{-1} m^{-1}. Dielectric constant determinations were usually carried out at pH 7.45, this being the pH value of the various aggregating media in which the cells were suspended. Measurements of specific conductivity were also carried out at the appropriate temperature in Radiometer CDM 2e conductivity meter with CDC 114 measuring cell (cell constant 6.5 mm^{-1}).

The compounds whose aqueous dielectric constant were estimated were obtained from various sources. The glycine diglycine and dextran were obtained from Sigma Chemicals Co., D-sorbitol was purchased from Koch-Light and Co. Ltd. and Ficoll (a polysucrose) was obtained from Pharmacia who reported an average molecular weight of 400,000. Solutions were made up in double glass distilled water and brought to the desired pH with 0.01 $mol\ dm^{-3}$ triethanolamine (B.D.H.) on a Radiometer 25 pH meter. Generally sodium hydroxide was not used to adjust the pH because its high ionic mobility interfered

with accurate measurements of capacitance above specific conductivities of 100×10^{-4} ohm^{-1} m^{-1}, reagents used were of "Analar" grade.

Tissue Culture.

(a) Materials: Hanks saline, calcium and magnesium free Hanks saline (CMF), diaminmethane tetracetate solution in CMF (EDTA), and suspension media were made up with the following composition.

Hanks saline - to make up one 1 dm^3, 8 gm NaCl, 0.4 gm KCL 1.0 gm glucose 0.35 gm $NaHCO_3$ 0.5 gm KH_2PO_4 1 cm^3 phenol red 0.14 gm $CaCl_2$ 0.1 gm $MgSO_4.7H_2O$ 0.1 gm $MgCl_2.6H_2O$ 3 gm Tris made up to one dm^3 in double glass distilled water with pH adjusted to 7.4 with HCl.

CMF saline - to make up one 1 dm^3, 7 gm NaCl 0.37 gm KCl 0.3 gm Na_2 $HPO_4.12H_2O$ 0.24 KH_2PO_4 1 gm glucose 3 gm Tris 2 cm^3 phenol red made up to one dm^3 in double glass distilled water with pH adjusted to 7.8 with NHCl.

EDTA - to the above ingredients for CMF saline add 0.001 mol dm^{-3} EDTA and adjust to pH 7.8 with NHCl.

Suspension Media - a 1:1 mixture of Hanks saline and Medium 199 (Flow Lab. Ltd.). Medium 199 was purchased as a 10x concentrate and is usually diluted to the normal concentration with double glass distilled water. In this investigation, the various compounds used to alter the dielectric constant were made up in the double glass distilled water before it was added to the 10x concentrate of Medium 199, the pH was then adjusted to 7.45 with NaOH. Control solutions of Hanks/

199 media were modified in a similar way by additions of D-sorbitol and NaCl to give equal pH, ionic strength and osmolarity to both experimental and control solutions. All manipulations in tissue culture were carried out under sterile conditions, and all solutions sterilized by passage through previously autoclaved millipore filters (Millipore Co. U.S.A.). All glassware and instruments were thoroughly washed and sterilized by heating at 160°C for two hours. "Nitex" cell sieves (Nitex, New York) were sterilized by autoclaving for 15 mins. at normal pressure.

(b) Methods: For each experiment one or two dozen fertilized hen eggs (Dekalb strain) were incubated at 37°C. On the appropriate day of incubation (7 days for Neural retina and 5 days for limb bud preparations) the embryos were removed aseptically from the eggs and placed in Hanks saline. The required organs were dissected out from the embryo into a separate dish containing Hanks saline at less than 3°C. From this stage onwards, great care was taken to keep all tissues used for experimental purposes below this temperature by using cold solutions. Tissues were disaggregated by the following technique (Curtis and Greaves 1965). The tissues were washed three times in CMF saline and then treated with 0.001 mol dm^{-3} EDTA saline for 7 minutes. After three further washings in CMF saline the tissues were mechanically disaggregated in CMF saline by repeated

gentle flushing through firstly a wide bore pasteur pipette and secondly a fine bore pipette. Care was taken in all cases to avoid over vigorous flushing and consequent bubbling of air through the suspension. The "cleaning" of cell suspensions was then performed. The cells were centrifuged at 25 g at 3°C for three minutes to sediment any cell clumps. A second harvest of cells was sometimes prepared from the pellet obtained from this centrifugation by re-suspending the pellet in CMF saline and reflushing through a pipette. The supernatant of the first centrifugation (or the pooled supernatants when a second harvest was made) was split into two equal volumes and then centrifuged at 300 g at 3°C for ten minutes to sediment the cells. The supernatant containing cell debris was discarded. The two pellets of cells were then re-suspended in known volumes of the required re-aggregating media one being defined control solution for the particular experimental medium under investigation. The cells were dispersed by use of a fine bore pipette and the media passed through Nitex cell sieves of 10um mesh size to ensure that the vast majority of small cell clumps were removed. The cell concentration was determined by haemocytometer and appropriate dilutions made to bring the total particle concentrations of experimental and control suspensions to equality. The cell suspensions were then either, transferred to couette viscometers previously prepared in a constant temperature room (2.5°$\pm$ 0.5°C) and aggregated

under identical conditions of shear for 35 minutes with regular samples being taken and transferred to haemocytometers for counting or, placed in the electrophoretic cell also previously set up at 2.5°C and the velocities of many cells observed in the stationary zones as will be described below.

5. Cell-Electrophoresis of Embryonic Tissues - theory

If charged groups are present on a surface, an electrical double layer is formed. It consists of an equivalent amount of charge of opposite sign distributed near the interface in the aqueous solution. This charge on the surface gives rise to an electrostatic field in the medium surrounding the particle. The potential of this field drops exponentially away from the surface. This affects adsorption to the surface and the interaction of particles in adhesion. The surface charge may be due either to adsorbed amphipolar ions or to the original structure of the surface.

If an electric field is applied to a system consisting of a solid and a liquid phase, there will be a movement of one phase relative to the other, at a velocity dependant upon the boundary potential across the plane of shear, the zeta potential ζ. Conversely, if one phase is moved mechanically relative to the other than a potential, referred to as the streaming potential, will be set up. The origin of these electrokinetic phenomena was first recognised by Quincke (1861) who

put forward the earliest concept of the electrical double layer. On this theory Helmholtz (1879) derived the relationship between the velocity, v, and the zeta potential

$$v = \frac{\zeta \in E}{4\pi\eta} \qquad (20)$$

where $\in$ is the electrostatic dielectric constant, η the viscosity of the medium and E is the field strength. Although the original Helmholtz theory involved no assumptions regarding the structure of the double layer, some general information on the distribution of ions at an interface is necessary to the understanding of processes occurring on the surface. The theory now accepted is that developed by Stern (1924) which embodied the principles put forward by Helmholtz and Guoy and Chapman. Helmholtz predicted that the double layer constituted a parallel plate condenser, but this was criticised by Guoy and Chapman on the basis that thermal energy would tend to distribute the ions throughout the solution. In place they postulated a diffuse ionic atmosphere in which the potential falls to zero the distance $1/\kappa$, the statistical thickness of the double layer. This simple picture predicts a capacity of the double layer higher than that obtained experimentally, the discrepency being attributed to the treatment of ions as point charges. Stern (1924) showed that alone

neither the sharp nor the diffuse double layer theory was adequate and he developed a theory embodying the essential characteristics of both. The double layer is thus divided into two parts; one approximately a single ion in thickness remains almost in contact with the surface, in this layer there is a sharp fall in potential. The second part extends into the liquid phase and is diffuse, thermal aggitation permits free movements of the ions. The distribution of positive and negative ions is not, however, uniform since the electrostatic field at the surface results in the attraction of ions of opposite sign, the charge on the surface is equal in magnitude but opposite in sign to the charges of the fixed and diffuse areas of the double layer.

A precise mathematical treatment of the relationship between the zeta potential and the migration velocity was not formulated for many years. Theories wereput forward by Helmholtz and Perrin and more recently by Debye Huckel and Henry. Smoluchowski (1903) regarding electrophoresis as the reverse of electro-osmosis derived the equation

$$v = \frac{\epsilon \zeta}{4\pi\eta} \qquad (21)$$

This equation can be applied to particles of any size shape or orientation provided they have "easy" shape and the radius of curvature of the surface was at all points

much greater than the thickness of the double layer. Debye and Huckel (1924) put forward a direct derivation of an electrophoretic equation from a consideration of the forces acting on a charged particle subjected to an applied field. Their equation is identical to that of Smoluchowski except for the constant. Their final equation which may be written

$$v = C \frac{\epsilon \zeta}{\eta} \qquad (22)$$

is valid independant of size and shape of the particle, but the constant C depends on both these factors. Analysis of the hydrodynamic equations for a sphere and a cylinder gave values for C as $1/6\pi$ and $1/4\pi$ respectively. Mooney (1931) and Henry (1931) independantly showed that the two theories applied to different limiting cases. While Smoluchowski assumed the radius of curvature of the particle was great compared to the double layer thickness, it was implicit in the treatment of Debye and Huckel that the radius of curvature was small compared with the thickness of the double layer. Debye and Huckel assumed that the applied potential was undisturbed by the presence of the migrating particle, an assumption only strictly valid when the conductance of the particle is identical with that of the medium or when the particle is so small that no appreciable distortion of the medium occurs in the double layer.

Since in general the conductance of the migrating particle can be taken as zero this theory applies only to very small spheres. Clearly then the mobility of particles under conditions intermediate between the two limiting cases will depend on the relative value of the radius of the particles and the thickness of the double layer. Generally the original Smoluchowski equation can be applied under the following conditions. (i) the radius of the particle is much greater than $1/K$; $1/K$ at 0.1 mol dm^{-3} ionic strength (approximately physiological) is 0.97 nm. This condition is easily satisfied in studies on tissue cells which have radii not less than 2 μm. (ii) the particle surface is non conducting, this condition is also fulfilled in the case of tissue cells where membrane resistance is of the order of 20,000 ohms per cm^2. (iii) the bulk values of dielectric constant (static) and viscosity are applicable in the double layer. Lyklema and Overbeek (1961) have discussed in detail the variation of both dielectric constant and viscosity in the double layer and its relation to electrokinetic potentials. They conclude that variation of dielectric constant is small at the field strengths normally found outside the slipping plane and in consequence there is likely to be only a small error in calculations of zeta potentials. The variation of viscosity in the outer part of the double layer is more serious, though not affecting the calculation of surface potential from zeta potential

the effect is to increase the value of zeta potential calculated by a given electrophoretic measurement by some 10% for cells in approximately 0.5 mol dm^{-3} ionic strength media.

In addition to these points when consideration is taken of the cell surface which may be crenated or folded instead of a smooth sphere it can be seen that the calculation of surface potential from electrophoretic measurements can be somewhat uncertain. But despite these more detailed points of criticism, electrophoretic data is of great value for the type of comparative study generally undertaken in biological research.

6. Cell-Electrophoresis of Embryonic Tissues - experimental

The apparatus utilised for this investigation was the Particle micro-electrophoresis apparatus MkII, consturcted by Rank Bros., Bottisham, Cambridge. Here the individual cells are followed under microscopic observation while under the influence of a known applied field. Facilities for both circular and rectangular cross sectional cells were available, in this study the cell of circular cross section was employed. The advantages of this cell are (i) small cross sectional area, resulting in a smaller current and less polarisation at the electrodes. In the present study no complications due to polarisation were apparent. (ii) Convection currents are minimal within this cell type. (iii) Heat exchange with the thermostatting bath is more efficient

in this cell than in the rectangular cell. It may also be noted that the thin-walled cylindrical cell provided has other advantages, over the frequently used thick-walled (Mattson) cells. The thick-walled cells must be ground down to a flat on the viewing side so that observations are made through a cylindrical plano-concave lens. This leads to uncertain optical corrections when locating the stationary levels. The major disadvantage of the cylindrical cell is that settling of large particles onto the lower wall of the cell may affect the electro-osmotic velocity over that wall and hence affect the symmetry of the cell and stationary layers. This was found to be no problem in the present studies since little settling occurred in the rather high viscosity media used. The electrodes provided were constructed of platinum and "blacked". Reversible electrodes e.g. Ag/AgCl, are also commonly used for electrophoretic measurements but platinum has the advantage of lasting for very long periods with no treatment except washing required. Also contamination of a biological system by toxic ions such as Cu^{2+} or Zn^{2+} is avoided.

The cylindrical cell was fixed at two points into a movable thermostatting bath. The cell was illuminated from the side by a quartz iodene lamp, focusing of the light beam onto the cell was accomplished by means of controls provided. A standard binocular microscope was used to observe the embryonic cells in the capillary

tube, the objective being water immersed. The cell was viewed from above using a 20x long working distance objective. A micrometer measuring to an accuracy of 0.002 mm was used to move the microscope up and down such that any level in the capillary cell could be focussed upon. Cleaning of the cell after use was accomplished "in situ"; after flushing with distilled water, a solution of Decon was left in the cell overnight and washed out thoroughly with distilled water the next day. Distilled water was kept in the cell until use to allow for complete leaching out of any water soluble chemical.

Calibration: The apparatus was provided with an electromagnetic timer which reads to 0.01 second. A voltmeter and ammeter are also provided, together with a thermistor used as the sensor for thermostatting purposes. The eyepiece graticule (squares of 10 by 10 units) was calibrated with the use of a stage micrometer (1 mm divided into 100 equal parts). As the objective in use was water-immersed, calibration was also performed with the objective in water, a simple petri-dish of water with the stage micrometer placed on the bottom was quite suitable.

The effective inter-electrode distance was determined from the resistance of the cell (at 25°C) when filled with a solution of KCl of a known specific conductivity at 25°C. The specific conductivity of KCl was measured using a Radiometer CDM 2e conductivity bridge with a

CDC 114 measuring cell. Using the relationship

$$L = RAK \qquad (23)$$

where L is the effective inter-electrode distance, R is the resistance of the KCl solution, K is the specific conductivity of that solution and A is the cross sectional area of the measuring cell, an effective inter-electrode distance of 82.77 mm was obtained for the cell.

Stationary levels: The velocity of a particle at any point in an electrophoretic cell will be the sum of the electrophoretic mobility and the velocity of the liquid caused by electro-osmosis (Kruyt vol. I. p. 219, 1952) which arises from the double layer at the glass solution interface. Electro-osmosis will cause movement of liquid towards one electrode, which will be balanced by a counterflow of liquid obeying the usual laws of viscous flow (Bangham et al 1958). Flow and counterflow combine to give zero liquid velocity at two levels. At these stationary levels the true electrophoretic velocity of the particles may be observed. It can be shown that the velocity Vl of the liquid at any level is given by (Kruyt vol. I. p. 219, 1952).

$$Vl = Veo\ (2r^2/a^2 - 1) \qquad (24)$$

where Veo is the electro-osmotic velocity, a the tube

radius and r the distance from the axis to the cylinder. At the stationary level where Vl = 0

$$r = \tfrac{1}{2} a \sqrt{2} \qquad (25)$$

This means that the electrophoretic velocity can be measured at a distance $\frac{1}{2} a \sqrt{2}$ from the centre or at a distance from the inside wall of 0.1465 x diameter of cell. For the present cell this corresponds to a distance from the inside wall of 254 um. With the thin walled cell provided, no optical correction was necessary to take into account refraction at the cell walls.

Experimental procedure: Tissue cell suspensions for electrophoretic studies were made up by the technique previously described (Tissue culture techniques). The electrophoresis cell was rinsed with distilled water then the cell and electrodes were further rinsed in the cell suspension. The measuring cell was filled to the top of the electrode sockets with cell suspension and the excess displaced by the electrodes so as to leave no air bubbles. The whole measuring apparatus was kept in a constant temperature room at 2.5°C $\pm$ 0.5°C, the thermostatting bath usually had less variability in temperature. The same part of the capillary was always used for both calibration and microscopical observation, this could be recognised by the pattern of scratch marks on the outer surface of the cell. Particles were timed

in alternate directions to minimise possible polarisation effects. Ten particles in each direction were timed for each stationary level, so that a total of forty readings were obtained in one experiment. Mobilities were obtained from the mean of the reciprocals of the times recorded. Where the mobilities in the upper and lower stationary zones differed by more than the experimental error the results were regarded as unreliable and discarded. It was discovered that using an applied voltage of 50 V, transit times could be kept within 5 to 11 seconds. This time scale is ideal for cell studies, errors in starting and stopping the timer loose significance over times greater than 5 seconds; whilst at times over 15 seconds there are greater chances of the cells moving out of the stationary level. Electrode effects (polarisation) were checked for by observing the ammeter reading with alternating polarity of electrodes. If changes in the reading do occur electrode polarisation is indicated. However, at voltages of 50 V used in this investigation this was never seen with any of the cell suspensions.

Viscosity of Cell Media

The viscosities of the various media used in the investigation were measured at 2.5°C, the same temperature under which the media were used for flocculation and electrophoretic studies. Use was made of an Ostwald viscometer, a simple capillary type which allows for

accurate measurements to be made. The viscometer was cleaned with Decon solution and washed repeatedly with double glass distilled water then dried slowly at 37°C. When dry the viscometer was brought to 2.5°C in a constant temperature room before use. Flow rates of the various media were then observed. The densities of the media were also estimated at 2.5°C with the use of a $10cm^3$ density bottle (Gallenkamp) which was cleaned and prepared in the same way as that described for the Ostwald viscometer.

RESULTS

Determination of Dielectric constants

(i) Resolution of measuring device

After preliminary trials with the measuring equipment described in Materials and Methods, it was found that the manufacturers specification for the B201 radio frequency bridge could not be easily met. In order to discover the true range of capacitance and conductance covered by the bridge, it was decided to test the resolution of the B201 on high quality standards. The Department of Electrical Engineering, Glasgow University provided, under the auspices of Dr. Foord, a General Radio type 1232-A detector tuned to 100 kH_3, a Sullivan variable air capacitor 70 pF to 1200 pF, and a Sullivan dual-dial non reactive decade resistance box. These standards were applied to the B201 bridge and the results of the test are shown in table (1). It can be seen that as conductance (reciprocal resistance) is increased the accuracy of the corresponding capacitance measurement becomes less, there being a gradual decrease in the measured value of capacitance to negative values as the conductance rises to higher values. Correct readings of both capacitance and conductance was limited to rather narrow bands of values, when either both capacitance and conductance values were high or low but never when capacitance was low and conductance

TABLE 1

Resolution of Measuring Bridge

Applied resistance and capacitance		Bridge balance	
R ohm	C pF	G μohm^{-1}	C pF
Bridge setting (a) Low x 100			
1000	1000	1002	1054.5
200	1000	5000	987.5
100	1000	9968	760.5
10 x 10	1000	9964	638.0
1000	100	1000	145.0
500	100	2000	144.0
200	100	4988	80.0
100	100	9945	negative value
Bridge setting (b) Block x 1			
1000	1000	1005	1064.0
500	1000	2006	1057.0
200	1000	5010	973.5
100	1000	10007	716.0
10 x 10	1000	10003	596.0
100	100	9962	72.0
10	100	no balance	negative value

high. This limitation of the measuring bridge meant that it was not possible to estimate a capacitance reading with any confidence when solutions of even moderate conductance were being tested, as the capacitance of the solutions under examination in this investigation tended to be rather low.

(ii) Calibration of measuring cell

Following the method described by Shaw (1942) the dielectric constant of a solution was calculated from measured capacitance by means of equation (19) where Co and K are constant for a particular dielectric cell. Calibration of the cell to obtain both Co and K was performed with the use of double glass distilled water and dilute solutions of KCl. A frequency of 1 MH_3 was chosen as the calibration frequency in order to avoid electrode polarisation effects. The parallel capacitance measured for a series of electrode spacings was given by

$$Cm = Co + Ke/d \qquad (26)$$

where Cm is the parallel capacitance measured, e is the dielectric constant of the solution under test, d is the electrode spacing and Co and K are the cell constants required. Co and K were evaluated by linear regression analysis from the straight line graphs of Cm plotted as a function of $^1/_d$. In order to obtain satisfactory results several calibration experiments were rumand a mean value for both Co and K obtained.

Originally, calibration was made over the electrode spacings 1.25 mm to 15 mm but owing to the slightly non-linear behaviour of capacitance with $^1/_d$ over this large distance that was observed for this dielectric cell, a narrower electrode separation distance was finally utilised, covering the range 1.25 mm to 6.25 mm. This final more accurate calibration led to values for Co and K of 0.98875 pF and 0.8389 pFcm respectively, the results of a typical experiment are shown in fig (5) and table (2).

In order to check the validity of the technique, the dielectric constants of water methanol and ethanol were measured. Values obtained for double glass distilled water at 20°C and 25°C were 80.45 and 78.40 respectively, at 20°C methanol had a value of 33.67 whilst at 25°C, 60% ethanol and 20% ethanol had dielectric constants of 48.00 and 69.40 respectively. These figures are in excellent agreement with those given in N.B.S.C. 514, N.B.S.C. 589, C.R.C. Handbook of Chemistry and Physics (1966). The mean figure obtained for water at 2.5°C was 87.14. After repeated studies at 2.5°C on the variation of the estimated dielectric constant of glycine with frequency and electrode separation, it was concluded that while over a wide frequency range (100 kH_3 to 1 MH_3) and electrode separation (any point within the calibrated range) the dielectric constant would fallwithin 5% of the theoretical value given by Wyman and McMeekin (1933),

Fig. 5. Plot of Cm against $^{1}/_{d}$

(results from table 2)
from linear regression analysis
Co = 1.0285 pF.
K = 0.8388 pF. cm.

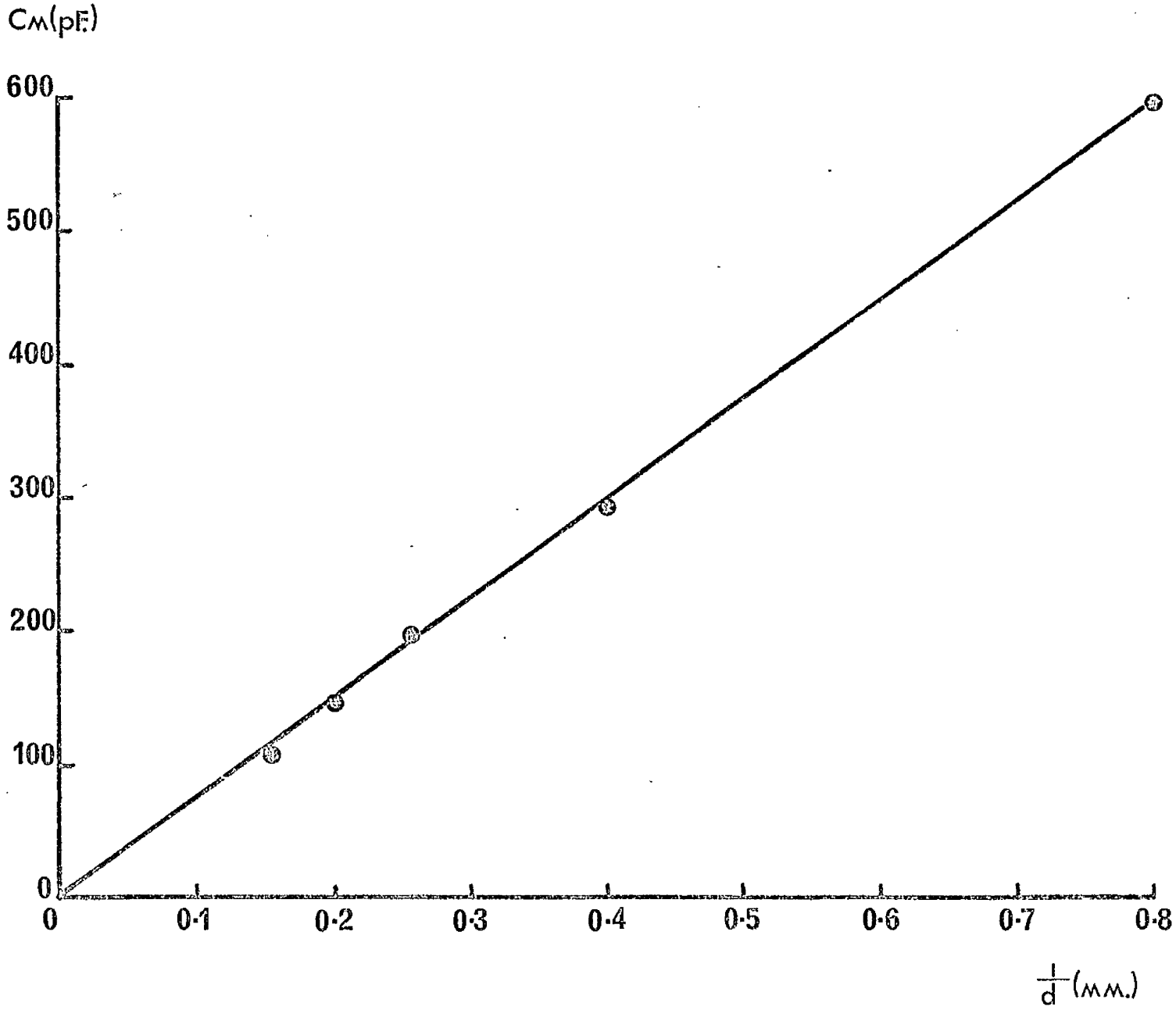


TABLE 2

Typical calibration of dielectric cell

Frequency: 1 MH_3

Temp: $2.5 \pm 0.5^{o}C$

Solution: Double glass distilled water: specific conductivity = 0.57×10^{-4} ohm^{-1} m^{-1}

electrode separation		capacitance	conductance
dmm	$1/d$	Cm pF	Gm μ ohm^{-1}
1.25	0.8	585.08	105
2.50	0.4	292.31	49.9
3.75	0.2667	195.5	32
5.0	0.2	147.19	24.1
6.25	0.16	118.11	20

Taking the dielectric constant of water as 87, (Handbook of Chemistry + Physics) and $Cm = Co + K\epsilon/d$

from linear regression

$Co = 1.0285$ pF

$K = 0.8388$ pF. cm.

an accuracy within 2% could be consistently obtained at electrode separation of 2.5 mm and 5.0 mm and a frequency above 200 kHz. The specific conductivity of the solutions employed gave the final limitations to the accuracy of measurement. Table (3) shows typical results for a glycine solution measured under conditions of variable frequency and conductivity.

(iii) Circuit induction

In order that the general approximations used to derive equations (11) (12) (13) (14) and (19) be satisfied, the induction, Lc, must be low (Mandel 1965). The inductance was therefore measured under the most unfavourable conditions to be encountered in the investigation, high conductivity. The technique used was the described by Schwan (1963 p. 375), aqueous solutions of increasing conductivity were measured at a fixed electrode spacing. Readings of Capacitance and Conductance were taken and from these the induction Lc can be obtained from (Schwan 1963)

$$Lc = \frac{d\ Cm}{d\ Gm^2} \qquad (27)$$

Lc being the slope of the straight line graph of Cm/Gm^2. In this way, inductances with values below 1.0 uH were obtained. The results of a typical experiment appear in table (4) and fig (6). These inductance values

TABLE 3

Variation of dielectric constant with frequency and conductivity

Solution: 0.5 mol dm^{-3} glycine in distilled water

Temp: $2.5 \pm 0.5^{o}C$

conductance varied with 0.01 mol dm^{-3} triethanolamine

Electrode separations: d_1 2.5 mm. d_2 5.0 mm.

theoretical dielectric constant 98.8 (Wyman and McMeekin)

Frequency	Specific conductivity (x 10^{-4} ohm^{-1} m^{-1})			
kH_3	<10	50	125	300
1000	98.82	98.31	98.95	106.95
500	98.84	98.76	99.22	105.20
200	99.74	100.05	98.29	-
100	98.90	99.40	100.10	-

TABLE 4

Circuit Induction LC

Frequency: 1 MH_3

Temp: $2.5^{o} \pm 0.5^{o}C$

Electrode separation: 5.0 mm.

Conductance varied with 0.1 ml dm^{-3} KCl in distilled water.

Specific conductivity ($\times 10^{-4}$ ohm^{-1} m^{-1})	Capacitance Cm pF	Conductance ohm^{-1} Gm (10^{-3})	Conductance ohm^{-1} Gm^2 (10^{-6})
223	137.55	4.396	19.32
307.7	127.40	6.203	38.477
361.5	118.25	7.485	56.025
507.7	86.40	10.644	113.31
638.5	49.20	12.988	168.68

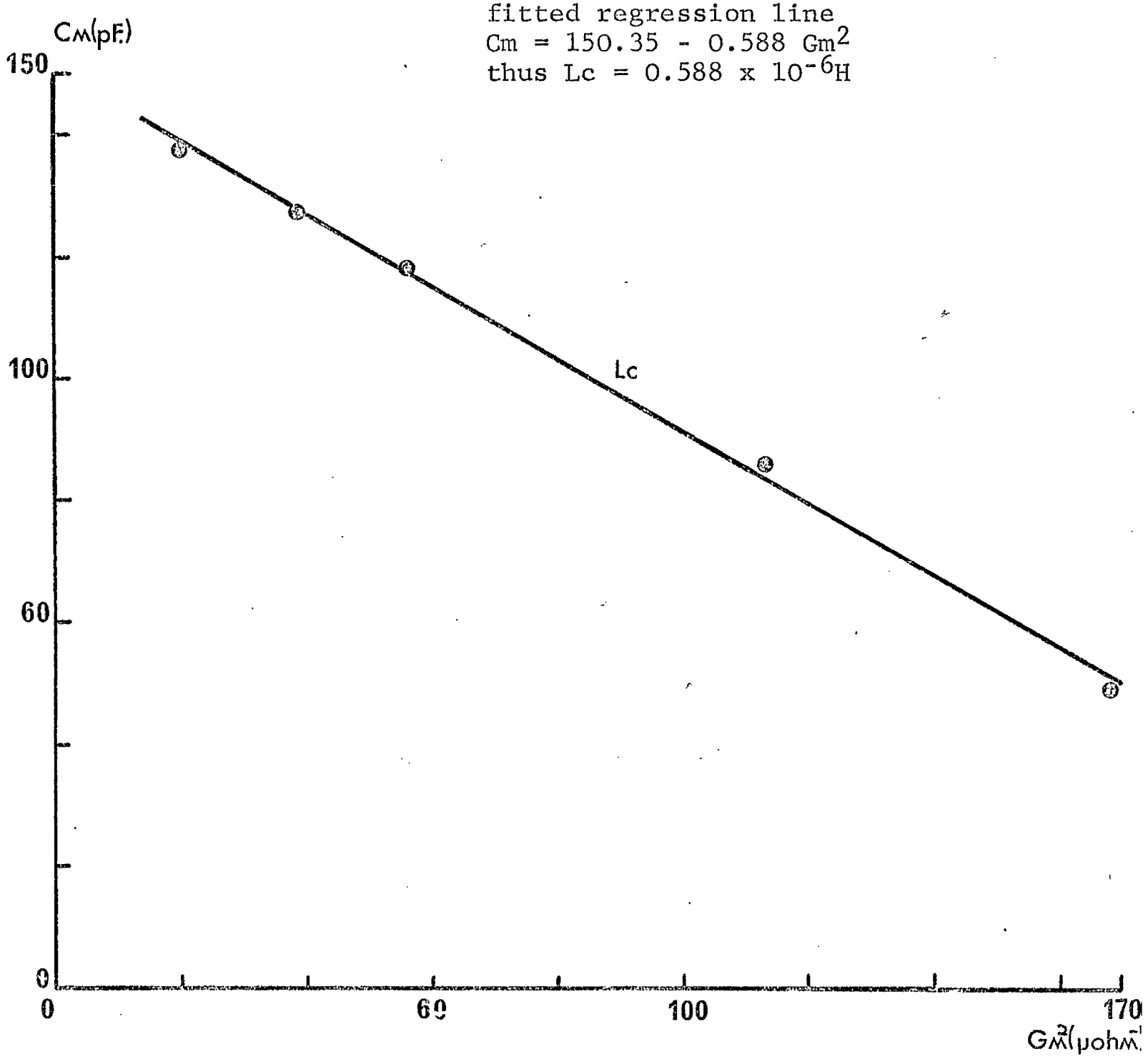
$$Lc = \frac{d\ Cm}{d\ Gm^2} \quad \text{(Schwan 1963 pp 375)}$$

thus from plot of Cm/Gm^2

$$Lc = 0.588 \times 10^{-6}\ H$$

Fig. 6. Plot of Cm against Gm^2

(results from table 4)
fitted regression line
$Cm = 150.35 - 0.588\ Gm^2$
thus $Lc = 0.588 \times 10^{-6} H$



fall within the range allowed for the approximations of the equations previously mentioned to hold (Young and Grant 1968).

(iv) Dielectric constants of experimental solutions

Because the conductivities of physiological media are far too high to allow for direct measurement of their dielectric constants (see Materials and Methods, Discussion), dielectric determinations were carried out on simple aqueous solutions of the various compounds investigated. All the solutions (except where specifically stated) were made up to the same "molarity" and pH as used in the electrophoresis and flocculation studies, the pH being adjusted with triethanolamine. This compound was found to be more satisfactory than sodium hydroxide, allowing for more accurate results with standard glycine solutions at higher specific conductivities. As suggested earlier (Materials and Methods) this finding may be due to the lower (ionic) mobility of triethanolamine, it being approximately three times less mobile than the Na^+ ion (Lawrence, A. J. private communication). Table (5) gives the results obtained for the solutions investigated in this study. Generally a frequency of 500 kH_3 was used for measurement, temperature was kept constant at 2.5°C ± 0.5°C in a constant temperature room. In all instances the dielectric constant of double glass distilled water was measured just previous to the estimation of the test solution. This acted as a check on the performance of

TABLE 5

Dielectric constant results

Temp: $2.5^{o} \pm 0.5^{o}C$

Electrode separation: d_1 2.5 mm: d_2 5.0 mm.

Specific conductivity of all solutions: $< 200 \times 10^{-4}$ ohm^{-1} m^{-1}

Each value a mean of at least 3 readings; variance < 2% in all cases.

Solution		pH	Dielectric constant	Frequency kH$_3$
Glycine				
0.1 mol dm^{-3}	unbuffered	6.4	89.48	500
0.1 "	buffered	7.45	89.31	500
0.5 "	unbuffered	6.2	99.02	500
0.5 "	unbuffered	6.2	99.25	100
0.5 "	buffered	7.45	98.60	500
1.0 "	unbuffered	6.1	111.5	500
1.0 "	buffered	7.45	110.7	500
Diglycine				
0.1 mol dm^{-3}	unbuffered	5.5	95.01	500
0.2 "	unbuffered	5.3	103.15	500
D-sorbitol				
5% w/v	buffered	7.45	86.57	100
5% "	"	7.45	87.17	500
5% "	"	7.45	87.10	1000
Dextran m. wt. 15-20,000				
2% w/v	buffered	7.45	86.22	500
5% "	"	7.45	85.60	500
5% "	"	7.45	85.20	100
5% "	"	7.45	85.50	1000
Ficoll				
1% w/v	buffered	7.45	86.70	500
5% "	"	7.45	86.09	500
5% "	"	7.45	86.08	100
5% "	"	7.45	86.09	1000

the dielectric cell and the values for the cell constants, Co and K, over the long period of use of the apparatus. Each value of the dielectric constant is a mean of at least three readings, usually taken in pairs. A typical dielectric determination is shown in table (6).

Microscopic examination of embryonic cell dispersions

On each occasion that cell suspensions were prepared for use in a study of either their flocculation behaviour or electrophoretic mobility in a previously unused aggregation medium, they were scrutinised prior to use under phase contrast microscopy (magnification x 400). The cells suspended in all the reaggregation solutions, except 1 mol dm^{-3} glycine media and the corresponding sorbitol control, were approximately spherical in shape. In the two media mentioned above the cells appeared very convoluted. The diameter of the cells was measured and found to be reasonably constant with a value for 5-day limb bud of 6.95 um $\pm$ 0.6 um S.E. and for 7-day neural retina of 5.90 um $\pm$ 0.8 um. These values are independant of reaggregation media. In all cases the cell suspension prior to use consisted of at least a 90% single cell suspension, the remainder being made up of cell doublets and triplets in an approximately 3:1 ratio.

TABLE 6

Typical dielectric determination

Estimation of 5% w/v Ficoll solution, m.wt. 400,000

Frequency: 500 kHz

Temp: $2.5^{o} \pm 0.5^{o}C$

Specific conductivity: 67×10^{-4} ohm^{-1} m^{-1}; pH 7.45

Electrode separation	mm	Capacitance pF	Conductance μ ohm^{-1}
d_2	5.0	142.95	1388.0
		143.00	1383.5
d_1	2.5	280.00	2763.0
		280.10	2752.0

Taking Co = 0.98875 pF and K = 0.8389 pF.cm

dielectric constant = (a) 86.096

(b) 86.060

The settling behaviour of the cells was also observed in all the solutions used. The cell suspensions were placed under a haemocytometer (Modified Fuch-Rosenthal) counting chamber and allowed to settle. At temperatures below 5°C it was found to take some twenty minutes for the suspensions to fully settle in Hanks/199, the least viscous of the media used. As the depth of the chamber is 0.2 mm, over the duration of the experiments (up to 40 mins) the particles would be expected to sink through a distance of 400 μm. Since this is less than 1% of the depth of the couette viscometers, it is unlikely that settling would affect the calculations of stability ratio through non-random distribution of cells in the aggregating medium. This rate of settling is also unlikely to have any significant effect on the electrophoretic mobility measurements, where rapid settling would quickly disrupt the original pattern of electroosmosis with unforseen changes in the depth of the stationary layers.

Cell viability was assessed by a plating out technique (Curtis 1969) to investigate the possibility of cell injury or death in any of the aggregation media used. The cells were plated out at very low population densities (ca. $1 \times 10^3/cm^2$) in the various media at 25°C ± 2.5°C. After one hour the proportion of cells which had settled and spread on the Petri-dish base was measured. Cells which had settled and taken up a normal morphological appearance were judged to be alive. This technique

probably gives an underestimate of viable cells because some cells which were alive probably did not settle and spread within one hour. Generally it could be seen that at least 65% and usually more than 70% were alive, but both for 5-day limb bud and 7-day neural retina, the percentage judged alive in 1 mol dm^{-3} glycine medium was less than 50%.

Measurements of viscosity

The viscosity of all solutions used in flocculation and cell electrophoretic studies was estimated at 2.5°C. Rates of flow for all the media were timed using the same Oswald viscometer, the same density bottle was similarly used for all measurements of density. Taking the viscosity of pure water as 1.6728×10^{-3} Nsm^{-2} (1.6728 cp) from C.R.C. Handbook of Chemistry and Physics, the viscosity of the reaggregation media were calculated from density and flow rate data. Table (7) gives the viscosities of the media used, each result is a mean of three measurements. The variation between these three readings was very low, differing by less than 2%.

Cell reaggregation in the couette viscometer

In all the aggregations measured, the plot of $\ln N_\infty t$ against t gave a straight line relationship, indicating that the aggregation followed the kinetics given in

TABLE 7

Viscosities of Reaggregation Media

Temp: $2.5^{\circ} \pm 0.5^{\circ}C.$

Media	Viscosity ($\times 10^{-3}$ Nsm^{-2})	
	Experimental media	D-sorbitol control media
Hanks-199	1.707	-
Hanks-199 0.1 mol dm^{-3} glycine	1.738	1.723
Hanks-199 0.5 mol dm^{-3} glycine	1.799	1.759
Hanks-199 1.0 mol dm^{-3} glycine	1.847	1.828
Hanks-199 0.1 mol dm^{-3} diglycine	1.7496	1.800
Hanks-199 0.2 mol dm^{-3} diglycine	1.805	1.847
Hanks-199 Dextran 5% w/v	3.238	-

equation (3), i.e. first order kinetics. The values for the collision efficiency given in Table (8) for 7-day neural retina and 5-day limb bud are all based on at least four experiments with six measurements of the total particle number at seven minute intervals. A typical example of such an aggregation is given in table (9) and the values plotted in fig (7) as $\ln N_\infty t$ against t. This indicated that the treatment of Curtis and Hocking (1970) is valid to these aggregations. Generally, when measurements were extended beyond 40 minutes the values for the stability ratio were seen to fall, indicating a departure from the kinetics of equation (3), possibly because of approach to equilibrium conditions. The appropriate shear rate chosen for aggregation studies differs between the two cell types, a rate of 10.24 sec^{-1} being used for the flocculation of 7-day neural retina, whilst 5-day limb bud were studied at a shear rate of 2.65 sec^{-1}. These shear rates were found by trial and error to be the most suitable values for the cell type concerned. During the course of the search for a suitable shear rate, it was noted that shear rate affected the value of the collision efficiency, such that an increase in shear reduced the collision efficiency obtained for cells in otherwise identical conditions. This observation agrees with that of Curtis (1969) for 7-day neural retina and confirms the applicability of the Curtis and Hocking treatment

TABLE 8

Estimation of Collision Efficiency (α)

Temp: 2.5°C ± 0.5°C.

Shear rate G sec^{-1} Neural Retina 10.24 sec^{-1} Limb Bud 2.65 sec^{-1}

each figure is a mean of at least 4 experiments

Aggregation medium	Cell Type: 7-day NR α %				5-day LB α %			
	Control	± S.E.	Experiment	± S.E.	Control	± S.E.	Experiment	± S.E.
Hanks-199	15.41	0.321	-	-	2.40	0.142	-	-
0.1 mol dm^{-3} glycine	15.26	0.254	16.80	0.322	2.30	0.134	2.86	0.202
0.5 mol dm^{-3} glycine	14.63	0.658	19.09	0.522	2.35	0.062	7.60	0.360
1.0 mol dm^{-3} glycine	16.24	0.868	22.54	0.748	1.33	0.456	5.89	0.543
0.1 mol dm^{-3} diglycine	14.93	0.431	18.36	0.219	2.25	0.084	5.075	0.204
0.2 mol dm^{-3} diglycine	14.99	0.297	19.73	0.309	2.47	0.067	7.35	0.092
Dextran 5% w/v (viscosity corrected)	14.96	0.325	11.04	0.377	-	-	-	-

TABLE 9

Typical Particle Count

Tissue: 7-day neural retina

Media: control media for 0.2 mol dm^{-3} diglycine Hanks-199

Temp: 2.5°C ± 0.5°C

G: 10.24 sec^{-1}

Total particle count.

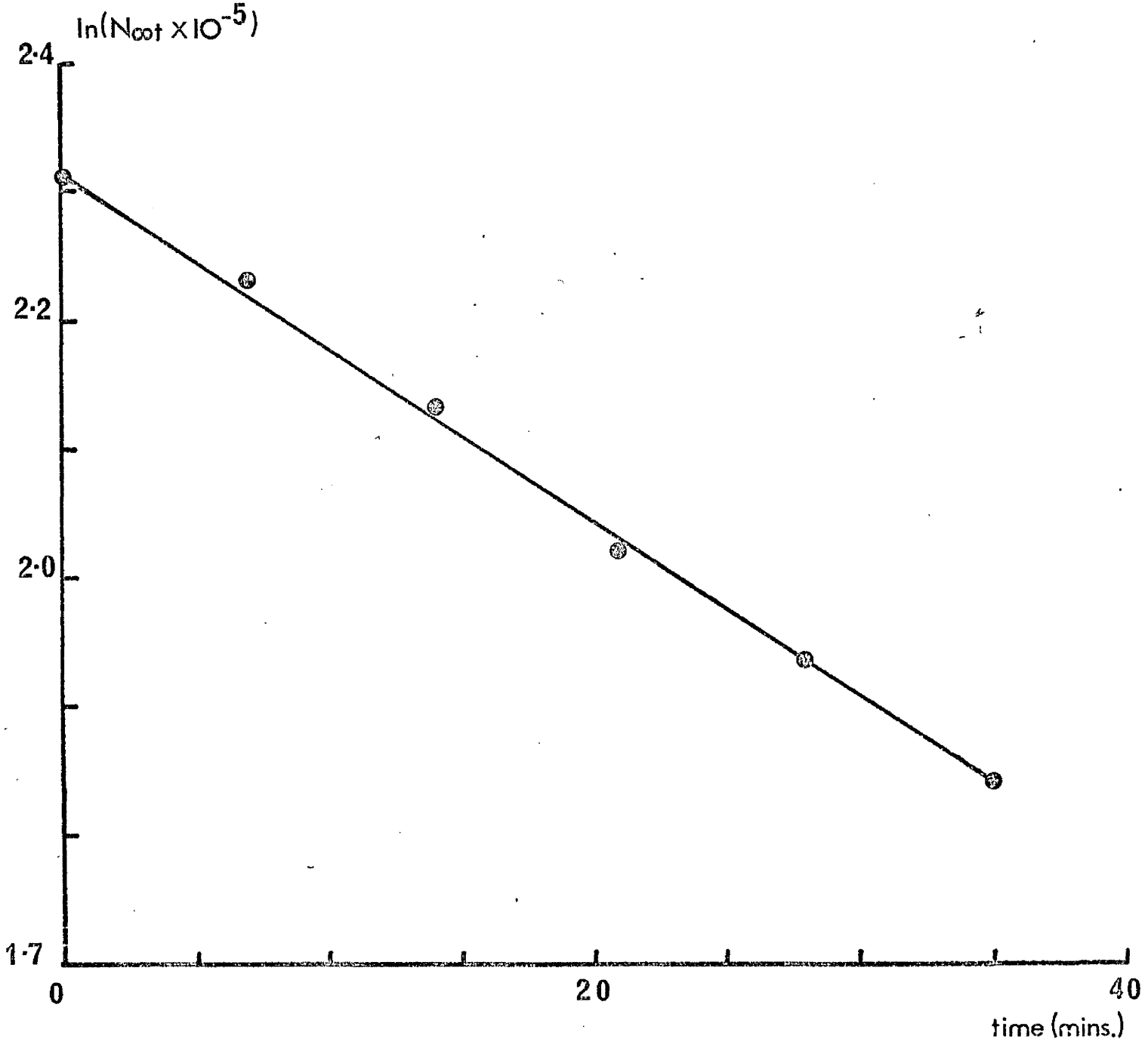
Time mins	Particle/ cm^3 x 10^{-5}	ln $N_\infty t$ x 10^{-5}	Collision efficiency α %
0	10.10	2.3125	13.23
7	9.30	2.2300	13.23
14	8.42	2.1306	14.58
21	7.54	2.0202	15.62
28	6.95	1.9387	14.98
35	6.30	1.8405	15.14

Mean 14.71
Variance 0.8233

linear regression: $\ln N_\infty t = 2.317 - 0.01365t$

Fig. 7. Plot of InN_{∞} t against time

(results from table 9)
fitted regression line
InN_{∞} t = 2.317-0.0136t



to these aggregations. From the collision efficiencies obtained by experiment, it is possible to calculate by means of equation (6) values of A, the London-Hamaker constant, or if no assumptions are made as to the nature of the adhesive force, equation (6) may be modified slightly to (Curtis 1969),

$$M = 72\pi n a^3 \, GH \qquad (28)$$

where H is a function of the stability ratio given by $H = 10^{1.178\sqrt{\alpha} - 10.86}$ and M is termed the force constant. Table (10) shows values for these parameters, calculated from a variety of cell aggregation experiments. Fig (8) presents a plot of the calculated force constants against dielectric constant of the suspension media for both cell types. The assumption that the dielectric constant of the media is equivalent to that measured in aqueous solutions will be discussed below. (see Discussion.)

Cell electrophoresis

Both 7-day neural retina and 5-day limb bud suspensions were examined under identical conditions of applied voltage (50V) and temperature (2.5°C). From particle velocity results, the mobilities were calculated and these were used to calculate zeta potentials using equation (21). Mobility and zeta potential results are shown in table (11), the zeta potential being corrected

TABLE 10

Calculation of Force Constants (M)

$M = 72 \pi n a^3 GH.$

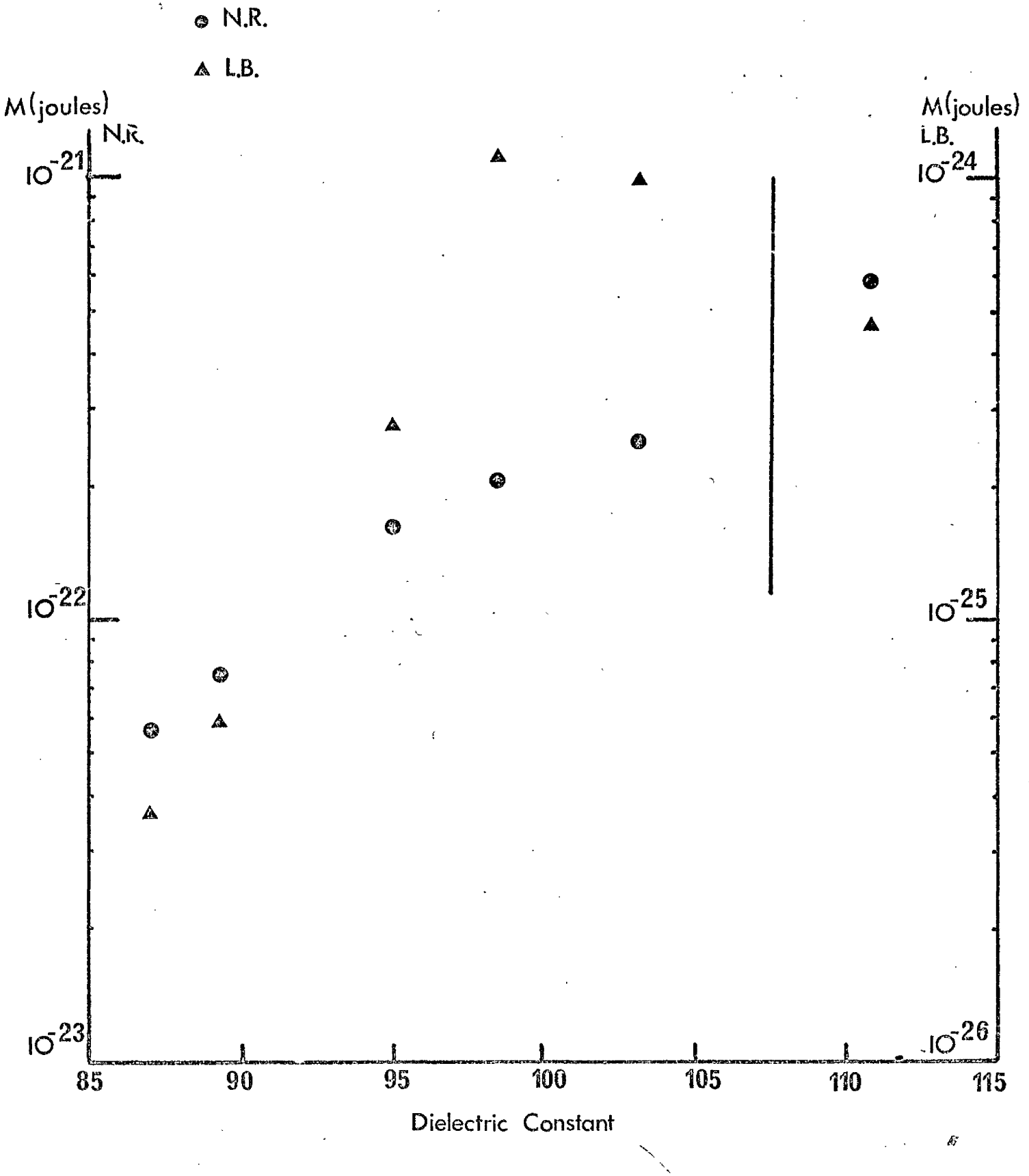
Aggregation media	α%	Interaction parameter H	Force Constant M joules
(a) Tissue type: 7-day neural retina: $G = 10.24\ sec^{-1}$			
Sorbitol control 0.1 mol dm^{-3} glycine	15.26	5.5158×10^{-7}	5.651×10^{-23}
Experimental media 0.1 mol dm^{-3} glycine	16.80	7.2913×10^{-7}	7.535×10^{-23}
Experimental media 0.5 mol dm^{-3} glycine	19.09	1.9338×10^{-6}	2.068×10^{-22}
Experimental media 1.0 mol dm^{-3} glycine	22.54	5.4163×10^{-6}	5.948×10^{-22}
Experimental media 0.1 mol dm^{-3} diglycine	18.36	1.541×10^{-6}	1.603×10^{-22}
Experimental media 0.2 mol dm^{-3} diglycine	19.73	2.3572×10^{-6}	2.529×10^{-22}
Sorbitol control 0.2 mol dm^{-3} diglycine	14.99	5.023×10^{-7}	5.516×10^{-23}

continued...

Table 10 continued

Aggregation media	α%	Interaction parameter H	Force Constant M joules
(b) Tissue type: 5-day limb bud; G = 2.65 sec^{-1}			
Sorbitol control 0.1 mol dm^{-3} glycine	2.30	8.451×10^{-10}	3.662×10^{-26}
Experimental media 0.1 mol dm^{-3} glycine	2.86	1.354×10^{-9}	5.918×10^{-26}
Experimental media 0.5 mol dm^{-3} glycine	7.60	2.440×10^{-8}	1.104×10^{-24}
Experimental media 1.0 mol dm^{-3} glycine	5.89	9.974×10^{-9}	4.633×10^{-25}
Experimental media 0.1 mol dm^{-3} diglycine	5.075	6.223×10^{-9}	2.738×10^{-25}
Experimental media 0.2 mol dm^{-3} diglycine	7.35	2.1568×10^{-8}	9.792×10^{-25}

Fig. 8. Variation of force constant with dielectric constant. Points above dielectric constant 110 are results for 1 mol dm^{-3} glycine media.



for dielectric constant and viscosity in equation (21). The mobilities and zeta potentials were estimated in a range of media used for cell aggregation studies, though the range was somewhat limited owing to the late availability of the equipment. Each mobility and calculated zeta potential given was a mean of at least three separate investigations, in which it was found that the variance was insignificant. Table (11) also shows values of the probabilities obtained when control and experimental media were tested by the Students 't' test. It was shown that while mobility results were significant to the 1% level, the corrected zeta potentials were not significantly different. The results obtained from a typical experiment are shown in table (12).

In order to check that calibration of the particle electrophoresis cell was adequate and that the equipment was being used correctly by the experimenter, the mobilities of human red blood corpuscles were measured. This material is recommended for calibration purposes (Seaman 1965) since it has been extensively investigated with the result that the electrophoretic mobility is known to a high degree of precision (Bangham et al. 1958). The electrophoretic mobility of washed human erythrocytes in 0.145 mol dm^{-3} sodium chloride solution with either added hydrochloric acid or sodium hydroxide solution is constant over the range of pH from 5 to 9 (Bangham Pethica and Seaman, 1958). In this study,

TABLE 11

Cell Electrophoresis

(a) 5-day limb bud tissue: zeta potential corrected for viscosity and dielectric constant. Each figure is a mean of 3 experiments.

Temp: 2.5°C ± 0.5°C

Voltage: 50 V

Suspension media	Mobility $um.sec^{-1}.volt^{-1}.cm^{-1}$	Zeta potential mV ± S.E.
Hanks-199	0.648 ± 0.002	-14.37 ± 0.031
0.5 mol dm^{-3} glycine H/199	0.6433 ± 0.0025	-13.23 ± 0.052
sorbitol control medium	0.5836 ± 0.0013	-13.32 ± 0.032
	$p = < 0.001$	$p = \gg 0.1$
0.2 mol dm^{-3} diglycine H/199	0.6621 ± 0.0015	-13.21 ± 0.09
sorbitol control medium	0.5625 ± 0.0134	-13.49 ± 0.32
	$p = < 0.01$	$p = \gg 0.1$

continued ...

Table 11 continued

(b) 7-day neural retina tissue: zeta potential corrected for viscosity and dielectric constant. Each figure is a mean of 3 experiments.

Temp: 2.5°C ± 0.5°C

Voltage: 50 V

Suspension media	Mobility $um.sec^{-1}.volt^{-1}.cm^{-1}$	Zeta potential mV ± S.E.
Hanks-199	0.626 ± 0.0018	-13.88 ± 0.04
0.5 mol dm^{-3} glycine H/199	0.6096 ± 0.0045	-12.54 ± 0.094
sorbitol control medium	0.5438 ± 0.0015	-12.42 ± 0.032
	$p = < 0.01$	$p = \gg 0.1$
0.2 mol dm^{-3} diglycine H/199	0.629 ± 0.0005	-12.29 ± 0.104
sorbitol control medium	0.522 ± 0.0019	-12.53 ± 0.049
	$p = < 0.001$	$p = \gg 0.1$

Mobilities and zeta potentials for control and experimental values compared by Students' 't' test. Probabilities given in the table.

TABLE 12

Typical Electrophoretic Results

Tissue: 5-day limb bud
Media: Hanks-199
Temp: 2.5°C ± 0.5°C
Voltage: 50 V

Graticule size: 29.5 µm
Interelectrode distance L: 82.77 mm
Field strength ($^{V}/_{L}$): 0.60408 V/mm

Each transit time given is a mean of two readings on one cell, the electrode polarity being reversed on each occasion.

	Transit time (secs)		Velocity (µm/sec)	
Upper	9.522	6.34	3.098	4.652
	7.04	6.51	4.190	4.531
Stationary	8.36	6.48	3.259	4.551
	11.59	8.08	2.545	3.650
Zone	6.34	6.83	4.652	4.319
			Mean = 3.945 µm/sec	
			mobility = 0.653 µm.sec^{-1}.volt^{-1}.cm^{-1}	
Lower	7.31	7.78	4.035	3.791
	9.68	9.631	3.047	3.064
Stationary	6.69	7.36	4.409	4.008
	6.14	8.59	4.820	3.434
Zone	7.73	6.71	3.816	4.396
			Mean = 3.882 µm/sec	
			mobility = 0.643 µm.sec^{-1}.volt^{-1}.cm^{-1}	

washed human erythrocytes were electrophoresed in 0.145 mol dm^{-3} sodium chloride solution at 25° ± 0.1°C buffered to pH 7, using the same manipulative techniques described in Materials and Methods for neural retina and limb bud cells. Calculated mobilities gave a value of 1.11 ± 0.03 um/sec/V/cm^{-1}, which was in good agreement with other figures quoted in the literature (Seaman 1965).

DISCUSSION

The interpretation of experimental results necessarily relies on making assumptions about the test systems used. In the investigations reported here there are a number of assumptions involved, those arising out of experimental measurements and those assumptions inherent in any theoretical treatment. In any work involving the measurement of physico-chemical parameters, an obvious basic assumption is the purity of the chemicals used. Generally one has to rely on the manufacturers, there is no reason to believe that they are untrustworthy. Further, in the case of the glycine compound obtained through Sigma dielectric measurements were carried out not only upon the compound as purchased, but also on purified chemical, the glycine having been re-precipitated in alcohol (Wyman and McMeekin 1933) before being dissolved in distilled water. No difference outside experimental error was seen between the treated glycine and the commercially available compound.

The three major assumptions of the theoretical treatment of cell adhesiveness as measured by the couette viscometers are (i) the cells are spheroidal, (ii) the suspensions are monodispersed, (iii) the cells have a density inappreciably different from that of the suspension medium. Optical microscopy has been used to provide information on the validity of these assumptions. Examination of the cells by phase contrast microscopy (see Results) indicated that generally the cells appeared

smooth and spheroidal at these levels of magnification, so the first assumption appears to be met. The second assumption that the suspensions consists of single particles of the same size can be borne out by the low standard errors of the particle size, whilst observations show (see Results) that at least 90% of the total initial particle count would be made up of single cells. Particle density was tested as described in the Results and from the observations made the third assumption can also be said to have been met. A fourth assumption implicit in the development of the theory of particle flocculation by Curtis and Hocking (1970) is that the particles (cells) are electrically neutral so that no appreciable electrostatic potential energy barrier exists. This was tested by cell-electrophoresis and the results for cell suspensions of both 7-day neural retina and 5-day limb bud tissue indicate an appreciable zeta potential. Since this is evidence of an electric charge on the cell surface, it appears that this fourth assumption cannot be met. However the assumption of electrical neutrality is not a necessary one, it being made for ease of computation (Curtis 1969), though the situation becomes more complex when the particles do possess an electric charge. The problems that this introduced into the treatment are dealt with later. While it would seem that the major assumptions made in applying the theoretical treatment to the experimental results are fulfilled in this system, it is essential to evaluate

the relevance of the theoretical approach. This requires that the results be shown to be consistent with the theory. In all aggregations, plots of $\ln N_\infty t$ against time were linear, which shows that the aggregations followed the kinetics predicted by equation (3), confirming the use of the equation for this system.

In the experimental measurement of dielectric constants, no major assumptions need be made. Calibration of the apparatus, and the demonstration of the validity of the measuring procedure by the results obtained on chemicals of known dielectric constant, is sufficient to predict that the experimentally observed dielectric constant of some other compound will also be correct. Two assumptions are made in relation to the theoretical meaning of the dielectric constants obtained, (i) the value of dielectric constants measured for solutions at low ionic strength (conductivity) will be the same at higher ionic strengths (conductivity), (ii) the radio frequency values obtained for dielectric constant will be equal to that at zero frequency, i.e. no dielectric dispersion will occur in the frequency range $0H_3$ to $1MH_3$. For reasons given in the Materials and Methods, it is not possible as yet to measure directly the dielectric constants of solutions of high specific conductivities. A compromise, used in this study, is to measure the value in distilled water, or better in an aqueous solution of appreciable conductivity and at a pH value similar to that of the cell suspension media.

In order to see whether any appreciable change in the measured dielectric constant could be detected with increasing ionic conductivity, values were taken over as wide a range of conductivity as the apparatus would allow. It is of interest to note that in the case of 0.5 mol dm^{-3} glycine for example, no regular change in the value of 98.6 for the dielectric constant at 500kHz can be seen, outside experimental variance, over a range of specific conductivities covering $< 10 \times 10^{-4}$ to $> 1.25 \times 10^{-2}$ ohm^{-1} m^{-1}. This result lends support for the first assumption, but in caution it must be remembered that the maximum value of 2×10^{-2} ohm^{-1} m^{-1} at which the dielectric constant was measured is nearly a factor 10^2 lower than the specific conductivity of the media met with in cell aggregation and electrophoretic studies. The value for Hanks-199 medium being 8×10^{-1} ohm^{-1} m^{-1} at 2.5°C. Hasted et al. (1948) have studied the dielectric properties of aqueous ionic solutions, they have shown that in solution concentrations below 2 mol dm^{-3} NaCl, a linear dielectric depression is found with concentration. This dielectric depression is greater at higher temperatures, at temperatures approaching 0°C the depression is of the order of only 10% for 0.66 mol dm^{-3} NaCl, the depression for this concentration of NaCl at 40°C being approximately 20%. The physiological solutions used in tissue culture techniques are generally made up to an ionic strength approximating 0.15 mol dm^{-3} NaCl, and this was also the case in the media used in this investigation. This point,

together with the choice of low temperature, was thought to be enough circumstantial evidence to assume that any reduction in dielectric constant at physiological ionic strengths would only be of the order of a few percent at most, compared to that measured at low ionic strengths on the radio frequency bridge. It may also be said that since the ionic strengths of the experimental and control mediawere made equal, any dielectric depression due to ionic concentration would be similar in both media, thus maintaining the difference in dielectric constant between both media. Turning to the second assumption, on the validity of high frequency measurements, it has been shown by Young and Grant (1968) that for glycine and diglycine a single relaxation time assumption is correct. They have measured the dielectric increments of glycine and diglycine solutions at a frequency of $7kH_3$ and found these to be in good agreement with the values deduced by extrapolation from decimeter wavelengths. A single relaxation time would allow for only a small increase in dielectric constant with approach to zero frequency, for example they quote an increased dielectric increment of 25.6 at $100kH_3$ to 25.9 at $7kH_3$ for α Alanine at 20°C. As for the other compounds studied here, no data appears to be available for the variation of dielectric constant with frequency, it was therefore initially assumed that since no appreciable change in the measured dielectric constants occurred with frequency (see Results) over the range measured ($100kH_3$ to $1MH_3$), no change occurred at

frequencies below 100kHz. This point will be further discussed below.

Considerable care has been taken during the course of this work to ensure that the cell suspensions used for experiment had been prepared and treated in an identical manner. In this way it was hoped that out of the total mass of cells originally excised from the embryonic tissue, the final yield would consist of a cell population with similar properties to that of cells harvested from the same tissue in some previous experiment. Several cell types do exist in both the tissues used, and if great differences were to be found in the porportions of these types from one experiment to the next, then consistent meaningful results would be hard to obtain, especially if the cell types happen to show differing adhesive abilities. Although consistency of cell population cannot be shown directly, it can be inferred from an examination of the collision efficiencies of the cells aggregated in the control media, and also in the electrophoretic mobility values of these controls. The generally low standard errors quoted in the Results would seem to indicate that at least as far as these two parameters are concerned, the cells obtained for separate experiments appear very similar in nature from one preparation to another. It has been previously shown (Results) that cells kept in 1.0 mol dm^{-3} glycine media and the control appeared to have low viability as tested by plating out

and/or appeared very convoluted in phase contrast microscopy. Viscosity of the media was not thought to account for the results of viability, as viability was high in the more viscous dextran-containing medium. Although the results of cell aggregation experiments using this medium are included, they may be rather inaccurate. The results show that the collision efficiencies for the control medium of 1.0 mol dm^{-3} glycine are also somewhat different from the average values obtained, it being a little higher in the case of 7-day neural retina and lower in 5-day limb bud. For both cell types, in control and experimental media the standard errors are greater than normal, all of which throws some doubt on the results obtained with this aggregation medium and its control. It is suggested that these results be ignored when considering the effect of dielectric constant on cell adhesion.

The figures obtained for dielectric constants generally agree well with the findings of other workers. The dielectric increment for glycine, quoted as 24.0 $\pm$ 5% at 5°C by Young (1967) and 23.6 at 2.5°C by Wyman and McMeekin (1933), compared favourably with that deduced from this investigation. The values obtained for glycine at 2.5°C fall within the range 23.1 to 24.5 regardless of measurement frequency or pH. Similar with diglycine the values obtained at 2.5°C vary from 80.1 to 80.75 compared with values of 84.4 $\pm$ 5% and 80.0 quoted by Young and Grant (1968). Diglycine was not buffered previous to

dielectric measurement, as at pH 7.45 the conductivity became too high for satisfactory measurements to be made. This discrepancy was not thought to be significant; as the results for glycine show, the addition of buffer made insignificant changes in the dielectric constant values measured, though it may be noticed that these values do seem to drop very slightly. Whether or not this is a property of the buffering agent triethanolamine is not certain, the question was not seriously investigated as the change in dielectric constant was too slight to cause concern. Measurements on aqueous solutions of D-sorbitol gave values of dielectric constant similar to water at this temperature. The results shows values for a 5% w/v solution of sorbitol buffered to pH 7.45. This solution was a far higher weight/volume concentration than that used for making up the control media (ca 1.5% w/v) and was used to exaggerate any dielectric effect. Over the frequency range covered the results show that D-sorbitol has no dielectric increment of measurable significance. Though no values for D-sorbitol could be found after a search of the literature, this result could be expected from a knowledge of the structure of the D-sorbitol molecule. It is a linear molecule obtained by a reduction of glucose, the formula is $C_6 H_{14} O_6$ giving a molecular weight of 182.17. This compound does not ionise in water, having a pH approximating 7 and an optical rotation $[\alpha]_D^{20}$ - 2.0 (Merck Index). The molecule is therefore not completely symmetrical and thus will have a permanent dipole therefore an aqueous solution of sorbitol may tend to reduce the

dielectric constant below that of water in the same manner as sucrose (Pethica 1961) though not so effectively. A very slight reduction in dielectric constant from the value of 87 for water may possibly be discerned at a frequency of 100kH$_3$, however this value is not outside experimental variance so the difference must not be stressed. What is evident is that the dielectric constant of D-sorbitol solutions are not increased above that of water. At frequencies approaching the static frequency the values will either remain similar to that of water, or if dielectric dispersion has occurred in the frequency range between static and 100kH$_3$, fall slightly to a lower value than that of pure water. The low molecular weight of D-sorbitol must argue against the latter possibility. Figures are also given in the results section for the dielectric constants for Dextran (mol wt 15-20,000) and Ficoll (mol wt 400,000) solutions. Broadly the two compounds exhibit similar behaviour with respect to concentration and measurement frequency. Both compounds show a decreased dielectric constant compared to pure water, dextran appearing more effective than Ficoll. Also in both cases, this decrease in dielectric constant is related to concentration, the higher the concentration the lower the dielectric constant. Both compounds appear to be unaffected by changes in measurement frequency in the range 100kH$_3$ to 1MH$_3$. In the case of Ficoll the whole range of values fall only very slightly below that of water and may not be significant while dextran may be

significant. The same general conclusion can therefore be made concerning dextran Ficoll and sorbitol solutions, that at frequencies at or below $1MH_3$, at the concentrations used here, the dielectric constants will remain similar to that of water. Dextran and Ficoll have rather high molecular weights however, and it is possible that dielectric dispersion may have occurred below the kilohertz range of measuring frequencies. Dielectric dispersion occurs when a molecule is no longer able to oscillate with the frequency of an applied a.c. electric field. If the molecule is too large, it may lag behind the applied field in its orientation, the higher the frequency of the a.c. field the more likely this is to occur. This dispersion leads to a lowering of the value measured for dielectric constant since molecules not in phase with the applied field will not register a complete dielectric effect. Thus for molecules of high molecular weight it cannot be simply concluded that the dielectric constant measured at kilohertz frequencies is the same as would be obtained at low frequencies approaching the static, as tacitly assumed earlier in the Discussion on the second assumption concerning the validity of dielectric measurements. If a dispersion had occurred below radio frequencies then a possibly large static value for the dielectric constant would not be recorded at kilohertz measuring frequencies. Two items can be introduced to support the belief that dielectric dispersion does not occur even in the Ficoll solutions of molecular weight

400,000. Schwan (1963) has tabulated the frequencies of major dispersions and it is shown that for protein and other macromolecular suspensions, no dispersion occurs below frequencies of 1 - 10 MH_3. It is also stated that where dispersion does not occur, the conductance of any solution will remain at a low level. Conductivity is a significant factor in the measurement of dielectric constant. If we consider a parallel plate condenser filled with a medium of given dielectric constant and conductivity, k, the equivalent shunt resistance is given by, (Edsall and Wyman 1958).

$$r = \frac{d}{A\,k} \qquad (29)$$

where d is electrode separation and A their area. At the same time the capacitance, C, is given by

$$C = \frac{A\,\epsilon}{4\pi d} \qquad (30)$$

by eliminating A/d between these two equations

$$r = \frac{\epsilon}{4\pi\, kC} \qquad (31)$$

Thus if large molecules do lag behind in an applied field, the effect will show up in the measurement as a conductivity effect. Generally a conductivity change of at least two orders of magnitude could be expected

(Schwan 1963). No such effect was ever seen in the investigation. For example, a glycine solution made up to a specific conductivity of 61.5 x 10^{-4} ohm^{-1} m^{-1} gave a measured bridge reading for conductance of 1312 x 10^{-6} ohm^{-1} at an electrode separation of 5.0 mm. Similarly a 5% Ficoll solution with a specific conductivity of 67.0 x 10^{-4} ohm^{-1} m^{-1} gave a reading of 1388 x 10^{-6} ohm^{-1} on the measuring bridge. The values can be considered equal if the slight difference in specific conductivity is taken into account. It is known (see above) that glycine shows no dispersion at these frequencies, and as no anomolous conductivity effect can be noted for the Ficoll solution under the same conditions of measurement, it can be assumed that no dielectric dispersion is likely to have occurred. It can be said that all the dielectric constant values measured in this study at frequencies between 100kH_3 and 1MH_3 can be extrapolated back to a static frequency value with some confidence. The specific rotation of Ficoll is $[\alpha]_D$ = + 56.5 (Pharmacia Ltd.) so that the polymerised sucrose though containing no ionised groups is not symmetrical and some permanent dipole will exist. Dextran with a rotation $[\alpha]_D$ = + 199 will also have a dipole. As with D-sorbitol and sucrose this dipole moment will have the effect of lowering the dielectric constant of aqueous solutions below that of pure water, possibly because the dipole moment per unit volume will be lower than that of water (Dr. A. J. Lawrence and Dr. Good, private

communications). The presence of this asymmetry may explain the slight reduction of the dielectric constant seen in aqueous solutions of dextran and Ficoll (Results). In none of the compounds studied except glycine and diglycine were there observed an increase in dielectric constant above that of water.

Pollack et al (1965) have also studied the dielectric properties of dextran and Ficoll solutions, and their findings are in complete contradiction to those given here. Studying aqueous polymer solutions adjusted to pH 7.45, they report dielectric increments for Ficoll and dextran of 14.70 and 40.45 respectively at 25°C. Utilising the figures of Pollack et al., the 5% w/v Ficoll solution should give a dielectric constant value of 152 at this temperature, while 5% dextran would have a value of 280.75. At the lower temperatures used for measurement in this study, the dielectric constants should have even higher values. No simple explanation can be given for these discrepancies in the results. Pollack et al. used a measuring frequency of 100kHz, a frequency also used in this investigation, so frequency dependant effects such as dielectric dispersion would not provide a solution. The molecular weight of the dextran used by Pollack et al. is not reported, but it is unlikely that it would be much lower, if at all, than that of the dextran used in this study. The molecular weight of Ficoll quoted by Pollack et al. is 100,000, however Pharmacia produce Ficoll at a molecular weight of 400,000, and as far as could be gathered, this was the only type they produce. If

Pollack et al. obtained Ficoll at a molecular weight of 100,000, it would have much the same properties as that of a molecular weight of 400,000, for reasons discussed earlier, at measuring frequencies in the kilohertz band. When at first no dielectric increments could be obtained, other lot numbers of the different polymers were tested, as Pollack et al. states that considerable variation was found between differing lots with respect to dielectric increment. Three other dextran lots and one other Ficoll lot were tested but in no case could there be recorded any dielectric increment of the type indicated by Pollack et al (1965).

It seems that the only explanation for the widely differing results can come from the measurement techniques, which differ in the two studies. The electrode distance variation technique used in this study is considered to be very accurate (Schwan 1963). However a constancy of polarisation impedence is necessary as the electrode spacing is varied. In practice it is desirable to follow the change in electrode position by a return to the original position, in order to check the original total impedence reading. The extent to which this can be successfully achieved reflects on the constancy of the polarisation impedence and hence on the accuracy of the technique. This simple check was always carried out and (as can be seen for the case of a typical dielectric estimation, table (6)) constancy of the results was always obtained.

The substitution technique used by Pollack et al. (1965) is reasonably successful if the elements which give rise to the unknown impedence properties (i.e. polymers) are of sufficiently low volume concentration to reduce the "shadow effect" to an insignificant level, or if this is not possible, to provide for this effect. The validity of the technique is based on the assumption that the polarisation impedence involved with the measurements of polymer samples and with contact fluid alone are identical. In many cases this is essentially true, but in others it may not be so, as shown by the investigations conducted by Bothwell and Schwan (unpublished, but discussed in Schwan 1963). Poor conductors such as cells and polymers can effectively reduce the electrode area which the current reaches ("shadow effect") and the polarisation impedence is increased because it is inversely proportional to the total electrode area reached by the current. Preparation of platinum electrodes, used by Pollack et al., can also have as yet unexplained effects with volume concentration (Schwan 1963). The presence of this effect in the work of Pollack et al. would account for the excellent values listed for glycine, water and ethanol, while giving anomolous results concerning the dielectric increments of the polymers investigated.

Brooks et al. (1967) lend support to the value of the dielectric increment for dextran obtained by Pollack et al. (1965). They were able to show that correction of the electrophoretic mobility value, for red blood corpuscles,

for the dielectric constant of dextran obtained by Pollack et al. gave mobility values almost independant of the dextran concentration. However, in a later communication (Brooks 1971) it was reported that the mobility and zeta potential effects are not due to any solution properties of dextran, but to counter-ion exclusion by the neutral adsorbed polymer layer. To this finding can be added the observation recorded in this investigation into the aggregation behaviour of 7-day neural retina cells in a dextran medium. The viscosity corrected value for the collision efficiency of this cell type is seen to be significantly lower in dextran medium than in the control. This finding may be explained by an increase in zeta potential, after the mechanism proposed by Brooks (1971), an increase in zeta potential will increase the repulsive potential energy of the cell surface and thus reduce their ability to aggregate (Gingell 1971).

Electrophoretic studies of tissue cells were carried out as a part of this study. However, the apparatus for cell-electrophoresis did not become available until the latter part of the investigation, so the amount of study involved is rather more limited than could be hoped. Experiments were carried out to test for the effects of glycine and diglycine, and the D-sorbitol control media on both 7-day neural retina and 5-day limb bud cell suspensions. Because of limited time, only 0.5 mol dm^{-3} glycine and 0.2 mol dm^{-3} diglycine concentrations were used these being thought to be the maximum concentrations at which the cells remained viable (see earlier Discussion).

Under the conditions of measurement used in this study (Materials and Methods), it can be seen from the Results that the calculated zeta potentials of 5-day limb bud and 7-day neural retina are similar, though neural retina tissue appear to have a slightly lower value, in a standard Hanks-199 medium. As far as can be determined, no other values appear in the literature for these cell types in the conditions used here, but the values for zeta potential agree well with other studies on embryonic cells in more artificial conditions. Collins (1966) obtained zeta potentials of -12.5 mV and -14.0 mV for 5-day chick heart and liver cells respectively, with 7-day neural retina lying somewhere between these values, (calculated from his mobility measurements). In all the other media in which measurements took place, the zeta potentials give slightly lower readings. The only obvious property held in common by all these media is an increase in the ionic strength above that of the standard Hanks-199 medium. Increased ionic strength should in theory reduce the zeta potential via a decrease in the thickness of the double layer and/or by neutralising the surface charge with cations (Kruyt 1952), and it may be that the general decrease noted here reflects this effect. Examination of the results show that the zeta potentials measured in both the experimental and control media are very similar. This evidence may be used to conclude that with none of the compounds used in cell aggregation studies, glycine diglycine or D-sorbitol,

does any significant adsorption to the cell surface occur. If adsorption were to occur, a large change in zeta potential would be predicted, presumably varying with the type of chemical involved (Ambrose 1966), also D-sorbitol has been used successfully before, by Heard and Seaman (1960) for measuring erythrocyte mobilities. If zeta potential is equated with surface potential, as is conventional though not strictly accurate, it can be said that the presence of D-sorbitol glycine and diglycine in tissue culture media do not significantly change the surface potential of the cell membrane either by adsorption or by any possible ion exclusion mechanism similar to that proposed by Brooks (1971) for dextran solutions. From theoretical considerations (Kruyt 1952) it is predicted that with an increase in the electrostatic dielectric constant of the suspension medium, the surface potential will change in value. Consideration of the relationship between the zeta potential and surface charge density of any spherical particle reveals that as the dielectric constant is increased, the zeta potential is decreased (Brooks et al., 1967, Weiss, L. 1967). The limited range of dielectric constant covered here is probably not wide enough to allow a serious appraisal of this effect, but it is noteworthy that in the results given for 0.5 mol dm^{-3} glycine and 0.2 mol dm^{-3} diglycine media, in all but one case the zeta potential is seen to be lower in the media of higher dielectric constant. The drops in zeta potential are however only slight (appx 0.2 mV) and are probably not

significant over such a small sample. Over the range of dielectric constants studied here it can probably be safely assumed that changes in the dielectric constant of the medium have virtually no effect on the zeta potential of the cell surfaces of either cell type investigated.

The values determined for the collision efficiency, from the aggregations of 5-day limb bud and 7-day neural retina are given in table (8) of the Results. These values can be used in two ways; as a means of calculating an absolute value for the force constant M, and as a way of comparing the rates of aggregation when these are carried out under similar conditions. If these conditions i.e. viscosity, shear rate and particle size are similar, it is clear from equation (28) that a comparison of the collision efficiencies is equivalent to a comparison of force constants. Hence it would be possible to compare the interactions of the cells under differing physico-chemical conditions without calculating the force constant in each instance. These conditions are fulfilled to a limited extent. Measurements of viscosity show that apart from the case of dextran-containing medium, there are no significant differences in the viscosities of the media used (maximum difference 0.124×10^{-3} Nsm^{-2}). The collision efficiency calculated for the dextran medium was corrected for viscosity separately before being included in the Results. The question of the radius of the cells is particularly important as the force constant is directly

proportional to the cube of the radius. Measurement of cell diameter in the various media (Results) showed that cell size did not differ significantly. The mean cell diameters of 5-day limb bud and 7-day neural retina are approximately 1.0um apart, which is not much greater than the experimental error. Considering the low variability of both these factors it is probably reasonable to use the collision efficiency to compare the adhesiveness within either of the two cell types. The difference in shear rate at which the two cell types were aggregated does not allow a direct comparison to be made between 7-day neural retina and 5-day limb bud, though comparison is allowed within each group owing to the constancy of shear rate employed for each type. It is therefore necessary to calculate force constants in order to be able to compare directly the adhesive behaviour of the two tissues.

The values for collision efficiency shown in table (8) demonstrate three overall factors in the aggregation of the cell suspensions, and hence it is presumed, in the adhesion of the cells. In all the experiments carried out, except for the case of aggregations involving 1.0 mol dm^{-3} glycine and its control media, the controls themselves give very similar results for collision efficiency, both within the neural retina and limb bud cell types. The consistancy obtained not only argues in favour of the whole test system being an excellent method for obtaining reproducible results, but also suggests that variations

found in the test suspensions can be confidently ascribed to real changes in the adhesive behaviour of those cells under study. It also serves to confirm that the assumptions concerning medium viscosity and cell radii are applicable. If viscosity was significant, it would be likely to alter the collision efficiency to some extent. This can also be said for cell size in conditions of varying "osmolarity", as it can be shown (Curtis 1969) that with two cells of equal adhesiveness a larger cell will be found to give the smaller experimental collision efficiency.

A second point arising from the results concerns the difference in the aggregation capabilities between the two cell types. The 5-day limb bud tissue in Hanks-199 medium at a shear rate of 2.65 sec^{-1} has a value for the collision efficiency of 2.4%, the 7-day neural retina cells at a shear rate of 10.24 sec^{-1} gives a value of 15.41%. This considerable difference in collision efficiency is seen to be even greater when shear rate is taken into account, it would be expected that 7-day neural retina aggregated at shear rates normally used for 5-day limb bud would provide an even greater value for the collision efficiency (Curtis 1969), probably somewhere in the region of 22%. The nearly tenfold increase in adhesive ability between 5-day limb bud and 7-day neural retina, as measured by this technique, might possibly be an indication of a difference in the type of adhesion that these two cell lines possess. This point will be discussed further below.

The third observation to be discussed is the behaviour of the cell suspensions when aggregated in media of differing electrostatic dielectric constant. In both cell types there is a tendancy for the cells agregated in the presence of D-sorbitol (the controls) to give collision efficiencies similar to that obtained in Hank-199 medium alone. This suggests the D-sorbitol has no effect on the aggregation of these cells and argues in favour of the experimentally observed evidence that D-sorbitol has no significant dielectric constant. In the case of neural retina tissue aggregated in a dextran containing medium, the collision efficiency (therefore presumably the adhesiveness) is seen to decrease. This result has been discussed before in relation to the work of Brooks et al (1967, 1971), but it may be added that it gives circumstantial evidence that dextran does not have similar delectric effects as glycine, which behaves in a completely opposite way. In both cell types there can be clearly seen an increase in collision efficiency (adhesiveness) in media containing glycine and diglycine, the only compounds measured here to increase the dielectric constant above that of water. It appears therefore that the adhesiveness of both cell types increases with electrostatic dielectric constant. To be able to examine this correlation more closely in the two cell types and to compare the effect between the two tissues, the force constants were calculated for a series of experimental results obtained in glycine and diglycine media, together

with one or two controls. These values appear in table (10). From these values the first thing to be seen is that large differences do appear between the two cell types as deduced from the force constnat. Taking as an example the force constant calculated for the D-sorbitol control medium of 0.1 mol dm^{-3} glycine, a difference covering nearly three orders of magnitude can be seen, neural retina having far higher values than limb bud tissue. This observation lends support to the possibility that the two cell types differ markedly in their adhesive properties. To clearly see the effect of dielectric constant on the adhesion of these cells, a graphical representation of the variation of force constant with dielectric constant is shown in figure (8). It appears that generally there is a steady increase in the force constant with measured dielectric constant, irrespective of the chemical (glycine or diglycine), while for the D-sorbitol controls, thought to have no dielectric effect, the force constants remain at the same levels (as can be deduced from the collision efficiencies). On closer examination of the results, it appears that 7-day neural retina is far less sensitive to changes in dielectric constant than 5-day limb bud. The former covers a range of values 5.6×10^{-23} joules to 5.9×10^{-22} joules if the result for 1 mol dm^{-3} glycine is included, and 5.6×10^{-23} joules to 2.5×10^{-22} if this glycine result is ignored. Limb bud on the other hand varies from 3.6×10^{-26} joules to 1.1×10^{-24} joules. At this juncture some difficulty arises in the correct

interpretation of the graphs in figure (8). If it is accepted that the results obtained for cell aggregation in 1.0 mol dm^{-3} are as valid as those in the other media, then it is possible to interpret the results as showing that 7-day neural retina cells show a linear increase in force constant with dielectric constant, while 5-day limb bud shows a gradual falling away as dielectric constant rises above ca 100. On the other hand, for reasons already mentioned, the results of experiments using 1.0 mol dm^{-3} are suspect, and therefore should be ignored. If this is the case then the interpretation of the results will be different. Namely, both cell types show increases in calculated force constants with dielectric constant, the rate of which appears to fall off when the dielectric constant rises above ca 95. It may be that both cell types appear to behave in a similar manner with increase in the dielectric constant, these increases becoming proportionally less effective in increasing the force constant.

The force constant, M, is a measurement of the total interaction energy acting on a pair of adhering particles. It may be equated with the Hamaker constant, A, if adhesion takes place in the primary minimum, when no appreciable potential energy barrier to close approach of the surfaces is found (Curtis 1969, Curtis and Hocking 1970). If the values of M derived from the measured collision efficiencies falls within the range of values for Hamaker constants deduced from other methods of measurement, it may be supposed that adhesion is occurring in the primary minimum, and that the London-Hamaker force of attraction is acting

in the adhesion being studied. Overbeek (1952) has shown that the Hamaker constant has values in the range of 1×10^{-22} to 1×10^{-19} joules (1×10^{-15} to 1×10^{-12} ergs). The values for the force constant, M, may fall outside the range of Hamaker constants for two reasons. The first of these is that the force of attraction is not the London-Hamaker force; however, there is a large body of evidence to show that this is unlikely to be the case (Curtis 1966, 1969). There appears to be adequate evidence that cells appear to act as colloidal particles (Curtis 1964, Born and Garrod 1968), and both Brooks et al. (1967) and Weiss (1968) have performed calculations showing that cell adhesion is explicable in terms of the DLVO colloid stability theory. The second explanation for a reduced force constant is that particle adhesion takes place in the secondary minimum because a potential energy barrier due to the presence of an electrical double layer prevents close approach of the surfaces. If adhesion takes place in the secondary minimum a low value will be found for the apparent Hamaker constant, represented here by the force constant, M. Study of the results for 5-day limb bud show that the values for the force constant are very low when compared with the values of Hamaker constant. From the results of electrophoretic studies (see table (11)) it is seen that this tissue type has a moderate zeta potential in the aggregation media utilised for aggregation studies. From theoretical considerations (Gingell 1971, Curtis 1967, Brooks et al. 1967, Jones, G. E. unpublished) it can be

Kappa.

shown that under these conditions a potential energy barrier will exist to prevent adhesion in the primary minimum. If the cells now adhere in the secondary minimum, a low value for the apparent Hamaker constant would be expected, and if this is equated with the force constant, M, this is what was found by experiment. It is suggested as a possibility that 5-day limb bud do adhere in the secondary minimum. In the case of 7-day neural retina tissue, the force constants calculated lie very close to the predicted Hamaker constants, though the values do appear to be on the extreme low side of the generally accepted values for Hamaker constants. From electrophoretic data (see table (11)) it appears that, under the same conditions, 7-day neural retina cells have a zeta potential comparable with that of 5-day limb bud. Using the same arguments as were applied above for 5-day limb bud, it could be supposed that this tissue also adheres in the secondary minimum. The higher values determined for the force constant may reflect either in a shorter separation distance between the adhering membranes of neural retinal cells, and/or in a higher true value for the Hamaker constant. This is known to depend on physical properties inherent in the bodies acting under the force of attraction as well as on the nature of the medium separating those bodies (Gregory 1969). However, it is possible that the force constant M, being strictly a measurement of the total interaction energy, rather than a direct measurement of Hamaker constant, must not be applied too rigorously when making deductions of this nature.

The effect of increasing the electrostatic dielectric constant in the work presented here is seen to increase the total energy of interaction of the cells when in the process of aggregation. From the results it can be seen that 7-day neural retina, with a greater interaction energy (M) than 5-day limb bud, is not so greatly affected by changes in the dielectric constant of the suspension media. This evidence seems to contradict all that has been proposed in theory concerning the effects of dielectric constant. From the theory of particle interaction it can be clearly shown that an increase in the electrostatic dielectric constant of the suspension medium will lead to an increase in the potential energy barrier, with an increase in the repulsive term of the total interaction energy. As a consequence there will be seen a decrease in the magnitude of the secondary minimum and an increase in the separation distance at which the minimum occurs (Brooks et al. 1967, Weiss 1967, Gingell 1971). Surface potential energy changes would produce correspondingly greater effects on the energy of interaction than would changes in the dielectric constant (Weiss 1967), since in computing the energy of repulsion, V_R, the square of the surface potential is used. Thus addition of any compound which lowered the surface potential would decrease the energy of repulsion and presumably allow the cells to aggregate more successfully in an enlarged secondary minimum. However measurements of zeta potentials of both cell types in high concentrations of glycine and diglycine clearly show little or no change from

the potentials obtained in control media. Furthermore, Gingell (1971) and Jones (unpublished) have shown by calculation that small changes (ca 2mV) in surface potential energy has very little effect on the secondary minimum, all of which suggests that possible surface potential effects may be discarded as an explanation of the results. Brooks et al. (1967) and Jones, G. E. (unpublished) have calculated curves of potential energy interactions with variable dielectric constant. They have shown that small increases in dielectric constant at a fixed Hamaker constant have very small effects on the total potential energy curves. One such curve (calculated by Jones) is shown in fig. (9) in order to indicate the magnitude of the effect. The dielectric constants used in experiment have covered a range from 87 to 103 (excluding the results for 1.0 mol dm^{-3} glycine) which is less than twenty units. The graph of fig. (9) shows curves where dielectric constant is varied over a similar range at a fixed Hamaker constant of 1 x 10^{-21} joules, with a surface potential of -20 mV. At a surface potential of -10 mV the secondary minima are deeper (ca 20 kT joules x 10^{7}) but the curves are of the same pattern. If these curves are now used to re-examine the results obtained from cell aggregation studies, some explanation can be arrived at. As indicated by fig. (9), it may be that the range of dielectric constant used for experimentation was too narrow to significantly effect the aggregation of cells into a possible secondary minimum, except when the media of higher dielectric constants were

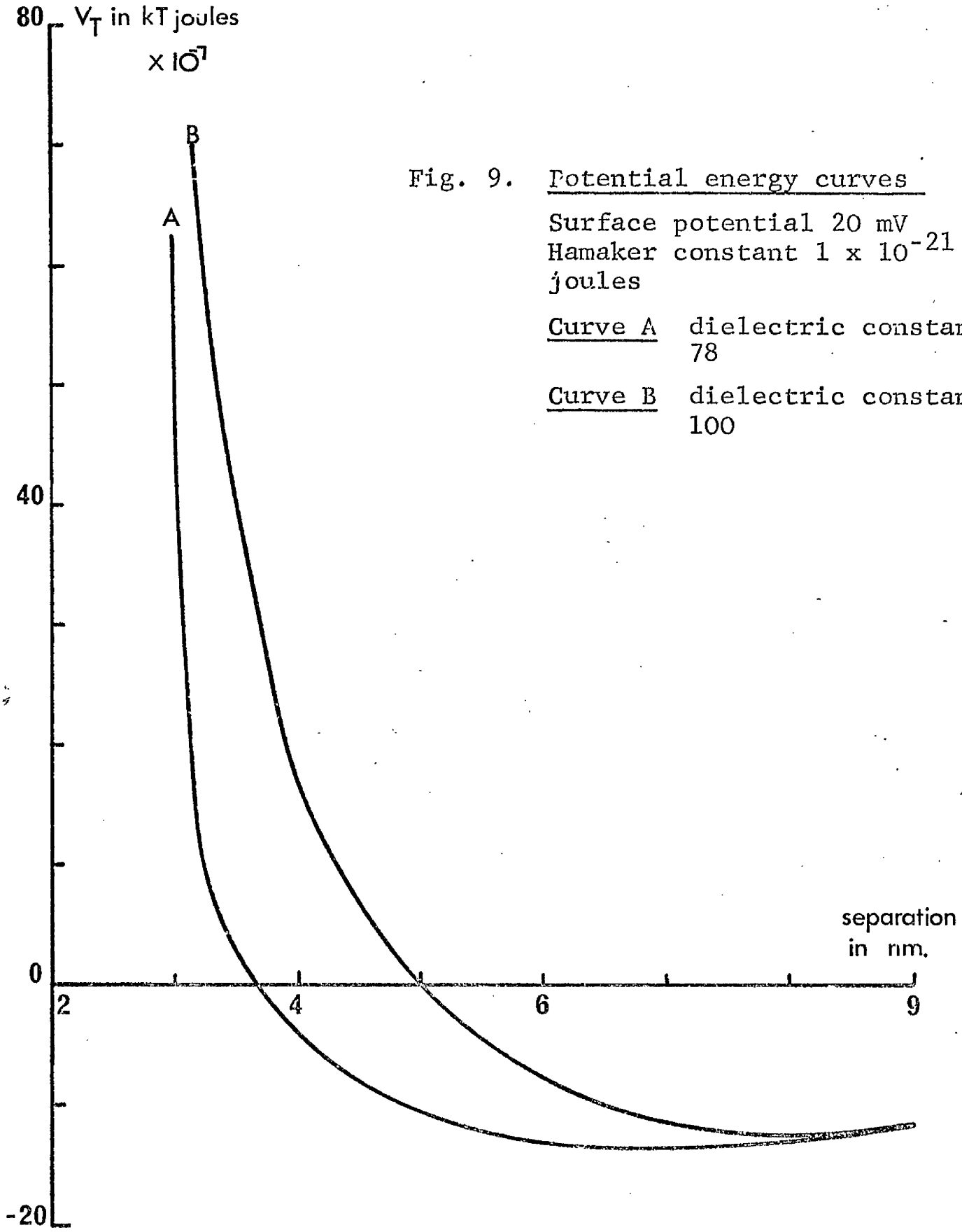


Fig. 9. Potential energy curves

Surface potential 20 mV
Hamaker constant 1 x 10^{-21} joules

Curve A dielectric constant 78

Curve B dielectric constant 100

being used, where the ability to aggregate (as measured by the force constant) begins to fall of somewhat (fig. 8), reflecting a significant decrease in the size of the potential energy "well" in the secondary minimum. While this would appear to be sufficient explanation for the observed falling off in the rate of increase in force constant with dielectric constant, it does not in itself explain why an increase should occur in the first place. In the early discussion, no attention has been paid to the possible affects of the addition of compounds such as glycine or diglycine on the energy or force of attraction, it being assumed that this was a constant value. Clearly, if the energy of attraction were to increase to a greater extent than the energy of repulsion, the total interaction energy would also increase; in line with the results presented in this thesis. The effect may exist in the limited range of dielectric constant investigated, where dielectric constant has not yet risen enough to significantly affect the repulsive energy, but where addition of quite large quantities of glycine or diglycine to the suspension media may have significantly altered the attractive energy.

It has previously been mentioned that the dielectric properties of the suspension medium can effect the energy of attraction (see Introduction). Lifshitz and his co-workers (Lifshitz 1956, Landau and Lifshitz 1960, Dzyaloshinskii Lifshitz and Pitaevskii 1960) have developed a theory of the molecular attractive forces between solid bodies in a liquid medium based on the idea of a fluctuating

electromagnetic field. The expression for the attractive force, which is derived from the Maxwell stress tensor is given in terms of the macroscopic dielectric constants and dielectric susceptibility of the interacting materials. In terms of the energy of attraction between two semi-infinite plates of material 1, separated by a small planar gap of medium 2, the expression is,

$$E = -\frac{\hbar}{16\pi^2 l^2}\int_0^{\infty}\left[\frac{\epsilon_1(i\xi)-\epsilon_2(i\xi)}{\epsilon_1(i\xi)+\epsilon_2(i\xi)}\right] d\xi \qquad (32)$$

where $\hbar = \frac{h}{2\pi}$, h = Planck's constant and $\epsilon_1(i\xi)$ and $\epsilon_2(i\xi)$ are the dielectric permeabilities of the two media evaluated on the imaginary axis in the complex frequency plane (Parsegian and Ninham 1969). The use of this method to calculate energies of attraction requires a knowledge of the dielectric data over a wide frequency range, except in the simplified case for distances of interaction greater than 200 nm where only the electrostatic frequency dielectric constant can be used. As the interactions under consideration are probably considerably less than this (ca 10 nm) it is not profitable to use this simple case. Dielectric data for aqueous solutions over a large frequency range are extremely limited as yet and certainly no comprehensive figures are available for solutions of glycine and the like, it seems that this approach is at present only useful when applied to simple water-lipid systems (Ninham and Parsegian 1970). A simplification of the theory can be derived by ignoring

contributions of the infrared to the van der Waals force of attraction and by using the optical frequency dielectric constant (which is the square of the refractive index). This approach would apply generally to transparent materials and should introduce only a small error into the calculation of Hamaker constants (Tabor and Winterton 1969, 1968). Recently Ninham and Parsegian (1970) have shown that, far from being able to ignore infrared contributions, it appears that these frequencies are much more important than was previously recognised, even so, Gregory (1966) when calculating the Hamaker constant from the refractive indices of the materials was able to obtain results for polystyrene in water that were in good agreement with the experimental value. Quantitative theoretical calculations based on the Lifshitz theory are not possible as yet in the case of the compounds used in this study, but inasmuch as the index of refraction of water changes with the addition of solute, so can the London-Hamaker force change. Thus by addition of glycine and related compounds it may be that the Hamaker constant is increased by some amount. If this were to be the case, as seems quite probable from a consideration of the Lifshitz theory, then for a given potential energy of repulsion, adhesions between particles would occur more readily, as the secondary minimum "well" is deeper at higher values of the Hamaker constant (Gingell 1971). It is suggested that the observed increase of the force constants seen in media containing glycine and diglycine

reflect just such an increase in the Hamaker constant. Possibly the large differences seen in the increase of the force constant between the two cell types may be a reflection on their differing adhesive energies (Curtis 1969), 7-day neural retina having rather more stable adhesions than 5-day limb bud (as measured by the collision efficiency) may not be so sensitive to small changes in the balance of interaction energies. The greater sensitivity of 5-day limb bud, producing large changes in the force constant, may be a further indication that this cell type rests in a shallow secondary minimum, requiring only small variations in the physico-chemical properties of the medium to alter its adhesive abilities to a large extent. As the D-sorbitol control media give rather consistent collision efficiencies, it would seem necessary to postulate that D-sorbitol does not exhibit the dielectric properties of glycine and diglycine which increase the attractive force. This seems quite possible, as Pethica (1961) points out that sucrose should have little effect on the attractive dispersion forces, but as with all that has been said on this effect, no quantitative data can be derived yet to show this.

One of the main objects of this study was to attempt to correlate the behaviour of aggregating tissue cells with the theories of cell adhesion outlined in the Introduction. By varying the conditions under which cell aggregation was observed it was hoped that evidence could

be obtained which would show a clear-cut preference for one theory or the other. Such a straightforward result has not been obtained, but the evidence does favour the theory of cell adhesion based on the colloid stability theory of Derjaguin and Landau: Verwey and Overbeek. The dielectric constant of the suspension medium does appear to have an effect on the adhesion of cells, a possibility predicted by the DLVO theory and not suggested by any other theory of cell adhesion. The effect is certainly more complex than was originally supposed. The apparent contradiction between what was expected by theory and what was experimentally observed can be explained by invoking an increase in the Hamaker constant, and consequently the attractive force. Generally in previous considerations of the physico-chemical factors acting in cell adhesion, the attractive force was either ignored or assumed a constant (Pollack et al. 1965), or taken into account by calculating potential energy curves over a series of differing Hamaker constants (Brooks et al 1967, Gingell 1971). The increase in the force constant with dielectric constant observed in this work may reflect a modification of the Hamaker constant brought about by the addition of certain compounds to the original medium. This can be visualised with the use of the Lifshitz theory, but as yet no pertinent calculations are possible. At a later date a further quantitative study may be attempted to make an appraisal of this phenomena in a model system such as lipid spherules (liposomes) in simple aqueous solutions.

To end the discussion on a more speculative note, it may be illuminating to consider in a quantitative sense the effect that the dielectric properties of an intervening medium can have upon the force of attraction between two bodies. For simplicity, the treatment of Dzyaloshinskii et al. (1960) for bodies at large separation distances, l, can be used, which utilises the static dielectric constant only. If both bodies are identical, the expression can be put,

$$F = \frac{\hbar c}{l^4} \frac{\pi^2}{240\sqrt{\epsilon_{30}}} \left(\frac{\epsilon_{10} - \epsilon_{30}}{\epsilon_{10} + \epsilon_{30}}\right)^2 \mathcal{Q}\left(\frac{\epsilon_{10}}{\epsilon_{30}}\right) \qquad (33)$$

where F denotes the force of attraction, c is the velocity of light and ϵ_{10} ϵ_{30} are the static dielectric constant values for the particles and intervening medium respectively. The function $\mathcal{Q}(x)$ has numerical values which are given in fig. (4) of Lifshitz (1956). This function has a limiting value of 0.35 which was used in these calculations. Taking an arbitrary value of $\epsilon_{10} = 50$, calculation with the use of eqn (33) gives a force constant of 3.56×10^{-27} J.mm./l^4mm when ϵ_{30} is taken as 87. When ϵ_{30} is raised to 107 (covering the range of dielectric constants measured in this investigation) then the force constant increases to 5.804×10^{-27} J.mm./l^4mm. In actual fact, the dielectric constant of cell membranes are much lower than the figure of 50 used here (Curtis 1967) so the effective increase in the force of attraction may be even greater. No calculations can be employed to show this however because

when the difference between ϵ_{10} and ϵ_{30} are greater than a factor 5, the function Q(x) no longer can be taken as the limiting value, but is increased greatly (see fig. (4) of Lifshitz 1956). This will cause even greater increases in F, but at present the increase in this function is a little uncertain making further calculation pointless. The force of attraction as given by eqn (33) is directly related to the Hamaker constant (Gregory 1969) and thus it can be seen from eqns (4) and (5) that the attractive energy between two particles will be increased by increases in F due to the dielectric properties of the intervening medium.

The use of eqn (33) has numerous disadvantages which have been mentioned earlier, but it does serve to show the effects of dielectric constant on the attractive energy rather clearly. It indicates that large changes in the attractive energy can be brought about by increases of the dielectric constant of the media in which particles (cells) are suspended, and would seem to support the conclusions reached in this discussion as to an explanation of the results obtained from cell aggregation experiments.

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