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A Study of the Flocculation of Phospholipids
as a Model of Membrane Interactions

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A Thesis Submitted to the University of Glasgow
for the Degree of Doctor of Philosophy.

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Contents

	Page No.
Summary	iii
Acknowledgements	vi
Introduction	1
Materials and Methods	21
Results	37
Discussion	44
Bibliography	79

Summary

Dispersions of the pure phospholipids phosphatidylcholine (lecithin) and phosphatidylserine were prepared in solutions of different ionic type and concentration. The dispersions were prepared so as to give particles, spherules, of the phospholipid which were of a fairly constant diameter, i.e. they were quasi-monodisperse suspensions. The dispersions were subjected to a laminar flow shear gradient in a Couette viscometer. The effect of this shear gradient was to effect collisions between the particles of the dispersions and thus to bring about flocculation or aggregation of the suspensions.

By measuring the total number of particles in the dispersions 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 can also be used to calculate the energy of the adhesive interaction of the particles.

The values of the collision efficiency for the flocculation of lecithin spherules showed that for the monovalent cations the order of increasing efficacy was Potassium: Sodium: Lithium which is the order of decreasing ionic radius. The results for divalent cations showed that these were more effective by at least two orders of magnitude. Further/

Further/

divalent cations exhibited a reversal of charge behaviour at concentrations above about 10^{-3} molar. The order of increasing efficacy did not reflect the order of decreasing ionic radius and was Magnesium: Strontium: Barium: Calcium, which suggests that lecithin may have a specific affinity for Calcium. The trivalent cation Lanthanum was more effective than the divalent cations and showed a charge reversal at a lower concentration. Temperature was found to have little effect on the rate of flocculation of lecithin dispersions.

For the flocculation of phosphatidylserine dispersions higher concentrations of sodium and calcium ions were found to be necessary, than for the flocculation of lecithin dispersions. This was to be expected because phosphatidylserine spherules are considered to bear a considerable negative surface charge, whereas those of lecithin are considered to be uncharged.

The collision efficiency, measured in ionic conditions where the spherules are probably uncharged, was used to calculate a value for the London-Hamaker constant. Values in the range 7×10^{-15} - 2.45×10^{-14} ergs were obtained for lecithin spherules and values of 4.7×10^{-15} - 1×10^{-14} ergs for phosphatidylserine. These values were used to calculate the adhesive energy of the particle interactions, assuming/

assuming/

that the particles adhered in the primary minimum with a separation of 5 Å. Values in excess of 200kT were obtained, indicating stable adhesions. These values are comparable with those derived experimentally and theoretically for other lipid systems.

As the spherules are considered to be made of structures which are similar to the cell plasmalemma and the spherules used in the flocculations have a size similar to that of cells, the result may be of interest in a study of cell membrane interactions, in particular cell to cell adhesion. The values of the London-Hamaker constant measured, would allow adhesion both in the primary and the secondary minimum as proposed in the lyophobic colloid theory of adhesion.

* * * * *

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INTRODUCTION

The cell concept of living organisms leads logically to the idea that a delimiting structure exists which separates a cell from the surrounding medium. From studies of permeation, interfacial properties and from electrical measurements, Danielli and Dawson (1935), Danielli and Harvey (1935), proposed an elaborated form of a model based on ideas put forward by Overton (1845) and Gorter and Grendel (1925). This model proposed that the delimiting structure was a membrane, of a basically lipoid nature, enclosing the cytoplasm. From the data then available, the paucimolecular membrane theory, as summarised by Dawson and Danielli (1943), was deduced. This theory proposed that the membrane, variously termed the "plasma membrane", the "plasmalemma" and the "cell surface" consists of a sheet of lipid molecules, two molecules thick, with polar groups directed outwards and the non-polar hydrocarbon chains oriented inwardly and perpendicular to the plane of the membrane. It was further proposed that the surfaces of this sheet, known as the "bimolecular lipid leaflet" are covered with/

with an adsorbed layer of protein.

The advent of the electron microscope allowed direct observations of the cell membrane to be made. Studies revealed a definite layer structure at the boundary of cells. Such studies, especially those on myelin, which was shown to be derived from the cell surface of Schwann cells (Geren 1954, Robertson 1957), when combined with earlier results from polarization (Frey-Wyssling 1953) and X-ray diffraction studies (Finean 1953, Fernandez-Moran and Finean 1957) tended to confirm the Danielli-Davson hypothesis. The electron microscope also revealed structures similar to the cell surface membrane in a variety of cellular organelles, which led Robertson (1959, 1967) to promulgate the "unit membrane" concept. This postulates that the major part of all membranes of all cells possesses the same basic structure, which is "a universal biological constant". The structure worked out for the membranes of myelin could, thus, be extrapolated to cover other membrane structures. The proposed structure is basically that of the Danielli-Davson model: a bimolecular leaflet of phospholipids covered with protein or mucoprotein or/

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 length apart of the hydrophilic moieties of two phospholipid molecules arranged in such a bimolecular structure (Robertson 1959). This typical triple-layered structure is best observed after permanganate fixation and has an overall width of about 75A.

From the data given by bulk analyses of myelin and erythrocyte ghosts, X-ray diffraction and the physicochemical characteristics of phospholipids (Finean (1957, 1966) proposed a model for the spatial arrangement of individual lipid molecules within the bimolecular leaflet. Vandenheuvel (1963a, 1965a, b) criticised and modified this model on steric and energetic grounds. This model takes as its starting point the fact that in myelin there is a 1:1 total phospholipid to cholesterol ratio. Vandenheuvel (1963), using Dreiding stereomodels and with maximum van der Waals' interactions as his touchstone, has shown that two basic stable structures/

structures are possible: the cholesterol-lecithin complex, characteristic of phosphatidyl lipids, and the cholesterol-sphingo myelin complex, characteristic of sphingolipids. Using these as a basic building block, it is possible to construct a three dimensional model of the bimolecular leaflet which has a phosphorus to phosphorus distance of about 50\AA . A figure which accords well with that derived from electron microscope and X-ray diffraction studies.

With such highly wrought models for the lipid bilayer, the structure of cell membranes appeared settled. However, the idea that there is a single universal structure for all membranes has of late been cogently criticised (Korn 1965, Lucy 1968, Chapman and Wallach 1968). The cornerstone of the "unit membrane" concept is the interpretation of the structure of myelin, a highly specialised component of nerves, and it may be questioned whether the composition of myelin is the same as other membranes. Elbers (1964) has shown that, in point of fact, the overall width of the triple-layered plasmalemmae, as observed in electron micrographs, can vary from about 50\AA to as much as 130\AA .

130Å. This may result from differences in the protein layer or may reflect differences in methods of preparation. The latter point is of great importance because in spite of the key position of the interpretation of electron micrographs in the analysis of membrane structure, there is still considerable dispute as to what information can be derived in terms of molecular structure from such micrographs (Korn 1966, Curtis 1967, Chapman and Wallach, 1968).

The interpretation of micrographs has been strongly influenced by the belief that the bimolecular leaflet is the most stable configuration adopted by phospholipids in water (Bevan and Malkin 1951, Dervichian, 1964). However, other configurations have been observed (Bangham and Horne, 1964; Lucy and Glauert, 1964). Luzzati (Luzzati and Husson, 1962; Luzzati, 1968) from X-ray diffraction studies has shown that a variety of structures exists for lipid water systems, dependent upon the physico-chemical conditions. He has suggested that the conditions extant in the living cell are close to those at which a phase transition may occur from a/
a/

a lamellar phase (bimolecular leaflet) to a phase with hexagonally packed cylindrical micelles with ionic groups directed to a core of water, may occur, the key parameters being water content and temperature. The possibility of such a phase transition makes the interpretation of electron micrographs even more problematical, though Stoeckenius (1962) showed that phospholipids prepared for electron microscopy at constant temperature exhibited the same structure as could be deduced from X-ray diffraction data.

The demonstration of an alternative phase state for phospholipids has led to the proposal that the phospholipids of the membrane may exist in such a configuration. A number of workers have obtained micrographs which would seem to indicate the presence of globular micelles in membranes.

Sjostrand (1963) and Sjostrand and Elvin (1964), found that mitochondrial membranes and smooth endoplasmic reticulum of mouse kidney and pancreas cells displayed globular sub-units separated by stained septa. Globular units have also been observed in membranes in sections of frog and tadpole/

tadpole retina (Nilsson, 1965, Blasie et al, 1965, Blasie and Worthington, 1968), in synaptic discs of goldfish (Robertson 1963) in chloroplasts (Park and Pen, 1963) and in mitochondria (Green et al, 1967).

A detailed model for a membrane constituted of lipid micelles, has been proposed by Lucy (1968) on the basis of observations of macromolecular lipid complexes using a negative staining technique (Lucy and Glauert, 1964). They have shown how a number of structures can be formed by the assembly of globular lipid micelles, in a manner analogous to that in which protein sub-units are assembled, as for instance in viruses. Lucy proposed that the membrane may be assembled from sub-units, in this case globular lipid micelles of about 40\AA diameter, in an approximately hexagonal array with a layer protein or glycoprotein on either side of the plane of micelles. This model envisages the membrane as a comparatively labile entity, with the micelles in continuous random movement about their mean position, with pores between some micelles and with the possibility of phase transitions occurring to the bimolecular leaflet configuration (Pethica, 1967; Lucy, /

Lucy, 1968). Another model which envisages transformations between equilibrium phase states to account for differences in membrane properties, has been proposed by Kavanau (1963, 1965). But there is as yet little evidence that such transformations do occur in living systems.

The once apparently unassailable bimolecular leaflet model has been frequently and stringently criticised of late and other models have been proposed. However, the weight of evidence would still seem to tip the balance in favour of the bimolecular leaflet model. It can be argued that much criticism of this model is a result of applying the bilayer model, in particular the "unit membrane" concept, with too great a rigour (Stoeckenius and Engelman, 1969). This model in fact, allows of greater variation than it is often credited with. The differences in membrane property and functions may result from the presence of specific proteins or peptides rather than structural differences (Mueller and Rudin, 1968). However, the data required to critically test any particular model of the cell membrane is not yet available, though new/

new techniques now being used may provide such evidence.

If our knowledge of membrane structure is limited, how much more so is that on the question of membrane interactions. Loewenstein (1967) has shown the importance of the interactions of membranes with one another and Gingell (1967) has speculated on the way in which membranes might interact. One field in which it might be thought that membrane interactions may be of importance, is that of cell adhesion. Curtis (1966, 1967) has reviewed the known facts on cell adhesion. From this, it would appear that there are two major classes of cell adhesion. The first, an adhesion with a separation of 100 - 200 Å between plasmalemmae, is relatively weak, of low specificity and is sensitive to ionic conditions, particularly the presence or absence of divalent cations. The second, the close adhesion, has a separation of less than 20Å and this adhesion may be specific with respect to cell type. These adhesions appear to be strong and, unlike the first type of adhesion, the cells involved cannot easily be/

be re-dispersed. Specialized membrane structures, such as desmosomes and zonulae occludentes also appear to be associated with cell adhesion.

Probably the oldest theory of cell adhesion is the cementing or bridging theory. This theory quite simply proposes that there is a cementing material in the gap between cells. The action of enzymes such as trypsin in dispersing cells, has been adduced as evidence in favour of a "cement" attaching to cells by means of covalent bonds. It has also been proposed that there are specific "cements" responsible for the adhesion of given cell types (Noscona 1961, 1962). There is no unenquivocal evidence for this theory or for that matter against it. The action of enzymes may be partly attributable to their action on extra-cellular materials such as collagen which have a strengthening rather than a purely adhesive role, and to possible alterations they may cause to the surface properties of the cell. Further, the evidence favours the view that the first type of adhesion (see above) is relatively weak and non-specific. While this is hardly a conclusive argument against the cementing theory, /

theory, it does tend to militate against the theory.

A specific mechanism, which is, in the main, a modification of the cementing theory, has been favoured by Pethica (1961) and Steinberg (1958, 1964). This proposes that calcium ions, thought to be of paramount importance in cell adhesion, can form ion-pairs or triplets with carboxyl groups on the cell surface (Haydon and Seaman 1962), thus acting as bridges between the cells. This model, as it stands, cannot accommodate the adhesion with a 100 - 200 Å separation, but it has been proposed that the calcium ions may "bridge" between the "cement" covering the cells, thus overcoming this problem. The dependence of cell adhesion on calcium ions, can be explained along other lines and there is little other direct evidence to support this theory.

One aspect of this theory as proposed by Pethica (1961) and Steinberg (1958) is that the cells are considered to be in molecular contact, the close adhesion (above). This view has been supported by the experiments of Wilkins et al (1962 a, b) which suggested that leucocytes flocculated at zero point of charge were adhering with molecular contact.

Weiss/

Weiss (1960, 1961) suggested the treatment of cell adhesion as a consequence of surface energy of contacting bodies, which also requires molecular contact. However, as stated above, there is a considerable body of evidence (Curtis 1967) which would seem to indicate that many cells adhere with a gap of 100 - 200 Å between the plasmalemmae. Certain specific cell to cell adhesions, such as sperm to egg and zonulae occludentes, may, on the other hand, be of this type. The evidence leads to the belief that a satisfactory theory of cell adhesion must allow for both types of adhesion.

Curtis (1960, 1962, 1966) has proposed that cell adhesion is a result of the balance between opposing physico-chemical forces. This theory draws on the theory of the stability of lyophobic colloids of Derjaguin and Landau (1941) and Verwey and Overbeek (1948). This theory visualises the main type of cell adhesion as a result of a weak reversible flocculation with the cell surfaces held 100 - 200 Å apart by a balance of adhesive and repulsive forces. It also allows for adhesion with molecular/

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, in this case cells, and of the gap material. The magnitude of the two forces falls off with distance, but the decrease with distance follows a different law in each case. The London force obeys an inverse power law and the electrostatic repulsive force an exponential law. The combination of these two forces predicts that when they interact two adhesions with 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 a few Angstrom units, (molecular contact). This is termed adhesion in the primary minimum. The second adhesion is a weak, /

weak, reversible adhesion with a separation 100 - 200 Å and is termed adhesion in the secondary minimum. The repulsive force is such that there is a large potential energy barrier preventing particles adhering in the primary minimum. The secondary minimum has no appreciable barrier to prevent the approach of distant particles.

Curtis (1967) has presented evidence that physiological conditions would favour a weak adhesion of cells in the secondary minimum, (see, however, Pethica 1961, L.Weiss 1967) with a separation ranging from 80 to 115 Å depending upon the values chosen for the London force constant and for the surface potential. Although the idea of adhesion occurring in the secondary minimum has been criticised, Schenkel and Kitchener (1961) provide good evidence that polystyrene particles so adhere. The theory thus predicts the possibility of two classes of adhesion: those with gaps of 100 - 200 Å between plasmalemmae, secondary minimum; and those with the surfaces in molecular contact, primary minimum. These correspond to the two classes of adhesion discussed above. Further, this theory predicts that adhesions in the secondary minimum will be relatively/

relatively weak and easily redispersed, as are the adhesions of many tissue cells. The ionic relations of the adhesions of tissue cells are correctly explained by this theory. High ionic strengths would be expected to reduce the surface potential and hence the electrostatic force of repulsion, thus promoting adhesion in the secondary minimum. Divalent cations are more effective in reducing the surface potential than monovalent cations, hence the importance of calcium ions in cell adhesion. The relative non-specificity of adhesion, whereby cells will adhere to a wide range of other cells and to many types of surfaces, is also a prediction of this theory. However, the enzymatic dissociation of cells cannot be adequately explained by this theory, save for the possibility of the enzymes altering the surface potential.

At present, the experimental data required to evaluate the various theories is not available. But the lyophobic colloid approach of Curtis (1966) seems to provide a more satisfactory explanation of most of the known facts of cell adhesion than the other theories. There is even less data on role of/

of the cell membrane in the adhesion of cells. There have been speculations about the action of specific molecules thought to be present on the lipid membrane (Weiss, L. 1960, Weiss, P. 1958) particularly about the significance of the carboxyl groups of compounds such as sialic acid (Cook et al 1961), but the role, if any, of the lipid component of the cell membrane has been somewhat neglected. As lipid forms the bulk of the interacting surfaces of adhering cells it would be of interest to know in what ways the nature and even phase state of the lipid might influence cell adhesion, and to have some idea of physico-chemical parameters of the lipids involved, such as their surface charge properties and the London - Hamaker force constant. Such parameters would also be of value in an understanding of the forces which give the membrane its stability and determine its structure. This thesis describes work designed as preliminary attack on this problem.

Till recently there has been no direct way of approaching this problem. Recently developed techniques have, however, provided an experimental approach. Models of cell membranes have been developed/

developed by a number of workers. Tobias et al (1962) used Millipore filter discs impregnated with phospholipids and Mueller et al (1962) formed bilayers of phospholipids, in water, analogous to soap films in air. However, the most useful model for the problem in hand is that developed by Bangham and co-workers (Bangham et al, 1965 a, b, Bangham 1968). They utilised the fact that phospholipids swell in aqueous solutions to form liquid crystals of the smectic mesophase type. These dispersions consist of approximately spherical particles of phospholipids, spherules, in the aqueous medium. From electron microscope studies Bangham et al (1965a) proposed that the spherules were composed of concentric shells of phospholipids separated by layers of water and that shells were in the form of bimolecular lipid lamellae, which, on general and thermodynamic grounds (Haydon and Taylor 1963), they suggested, are completely closed structures. Lucy (1968) has questioned this interpretation, but there seems to be little evidence to support his view that the shells are not concentric and contain globular micelles which provide/

provide access from the surrounding aqueous medium to the inner layers. The spherules would appear to be a not unreasonable model of the cell surface membrane and as they can be of comparable dimensions to cells it seems feasible to use them as a model of adhering cells.

The second technique utilised is one which was developed to allow an absolute measurement of cell adhesion to be made. Measurements have been made previously of the force required to separate cells (Dan, 1936, Brooks et al, 1967). These can be criticised on a number of grounds: that inter-cellular materials such as collagen may contribute to the resistance of cells to dispersion and that the force measured may merely be that required to rupture cells (L.Weiss 1961). Because of these difficulties, attempts have been made to measure adhesiveness during the formation of cell to cell adhesions. Moscona (1961) used the size of aggregates, formed when a suspension of single cells was shaken, as a measure of adhesiveness. It is difficult to derive an absolute measure from such results.

Curtis/

Curtis (1969) has described a technique which allows such a measure to be made from the rate of aggregation (flocculation) of particles (cells). The technique rests on two basic concepts. First, when particles in suspension are brought into collision by shaking, the movements of the medium bring the particles together and then, as the particle which travels more rapidly passes the other, tend to re-separate them. A stable adhesion will form, if the adhesive force is great enough, to bring the particles close enough together, to resist the subsequent break-up of this newly formed aggregate. The second concept is that the proportion of collisions which effect an adhesion is a measure of adhesiveness. This proportion is termed the collision efficiency or stability ratio. It has been measured previously, though seldom by a direct method, and Fuchs (1934) derived an absolute measure of adhesive energy of particles from the stability ratio. However, this treatment is not really applicable to shaken suspensions of particles such as cells, because it treats the/

the particles as coming together by Brownian motion whereas in shaken suspensions much larger hydrodynamic shear forces are acting. Also it assumes that a potential energy barrier is present preventing collisions resulting in adhesion and in the case of cells this is probably not true (Curtis 1969).

Curtis and Hocking (in press) have developed a new technique for deriving the adhesive energy from the collision efficiency, which is applicable to measurements made in shaken (sheared) suspensions (see Material and Methods section). This technique has been used in the study of the flocculation of dispersions of the phospholipid lecithin, which was chosen because it is a major constituent of the few membranes which have been analysed (Dawson et al 1960, Rouser et al 1968). In this way, measurements have been made under a range of ionic conditions to determine the interactions of lecithin spherules under such conditions and also to gain an absolute measurement of the adhesive energy of these particular spherules.

MATERIAL AND METHODS

(i) Method for Determination of Particle Adhesiveness - Theory

In a suspension of particles, collisions are brought about either by Brownian motion or by movements of the medium. If, in the latter instance, the movements are such that the particles are brought into collision by a laminar shear gradient, of rate G , it is possible to derive a theoretical frequency for collisions between particles. Von Smoluchowski (1916) showed that for particles of radius r_i and r_j , at concentrations n_i and n_j , the total number of collisions per unit time interval, b_{ij} , during a flocculation or aggregation in a laminar shear field is given by the relationship

$$b_{ij} = \frac{4}{3} G n_i n_j (r_i + r_j)^3 \quad (1)$$

where G has the dimensions of reciprocal time.

This equation cannot be applied to the whole course of an aggregation because once an appreciable number 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 function by integrating the/

the total number of collisions for all classes of aggregate size. They showed that this size distribution function is mathematically consistent with the Von Smoluchowski theory of coagulation and that the total number of particles at time, t , $N_{\infty t}$, compared with those at the start of aggregation, $N_{\infty 0}$, is given by

$$\ln \frac{N_{\infty t}}{N_{\infty 0}} = - \frac{4 G \phi t}{\pi} \quad (2)$$

where G is again the shear rate and ϕ is the volume fraction of particles in the suspension. On the other hand, it should be noted that Fair and Gemell (1964) concluded that the Smoluchowski equations for orthokinetic flocculation, i.e. under conditions of shear, could not be solved analytically.

Movements of the medium will, however, 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

$$\ln \frac{N_{\infty t}}{N_{\infty 0}} = - \frac{4 G \phi \alpha t}{\pi} \quad (3)$$

where α is the collision efficiency.

The collision of particles which are small, in the sense that the Reynolds number for their motion in the fluid 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 (Drenner 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 result in an adhesion occurring during a collision and hence lead to the formation of doublets, 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/

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, which varies inversely as the sixth power of distance between the molecules. At appreciable separations between molecules, when the distance is comparable to the wavelength of the London frequency the force will be smaller than that given by the simple relationship and the attractive energy, when allowance has been made for this retardation factor, has been calculated by Casimir and Polder (1948). 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/

to the distance between them, the attractive energy V_a (attractive energies are conventionally negative and repulsive energies positive) can be simplified to

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

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

$$V_{aH} = - \frac{Aa}{12 H} \quad (5)$$

with, a , 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 (1968). As the energy of London forces at small gaps 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 and doublets will be formed.

Curtis/

Curtis and Hocking (in press) 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. Treating 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:- (1) the effect of the shearing motion on the particles, (2) the hydrodynamic forces, (3) 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

$$A = 72 \pi \eta a^3 G K \quad (6)$$

where η 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 the 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 suspension, can be applied to adhesions in the primary minimum, provided 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 computational difficulties in this case. If a potential energy barrier exists, Fuchs' treatment (Fuchs 1934) is applicable. The treatment of Curtis and Hocking shows that the collision efficiency is dependent on the shear rate, equation (6). Whereas Fuchs' treatment has as its rate-determining step, the frequency with which Brownian motion energy exceeds a certain value, allowing the potential energy barrier to be surmounted. This is not dependent on shear rate and the collision efficiency will thus be independent/

independent of shear rate. This provides a method of assessing the applicability of the two treatments. It may eventually be possible to modify Fuchs' treatment so that flocculations in a shear gradient with a potential energy barrier may be dealt with.

(ii) Method for Determination of Particle Adhesiveness - Experimental Approach.

The relationship given by Swift and Friedlander (1964) (Equation (3)) allows the collision efficiency for the aggregation 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 (G), 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 is rotated. Laminar shear flow is established, with a shear gradient across the gap, and the shear rate, $G \text{ sec}^{-1}$, is given by

$$G = \frac{v}{r}$$

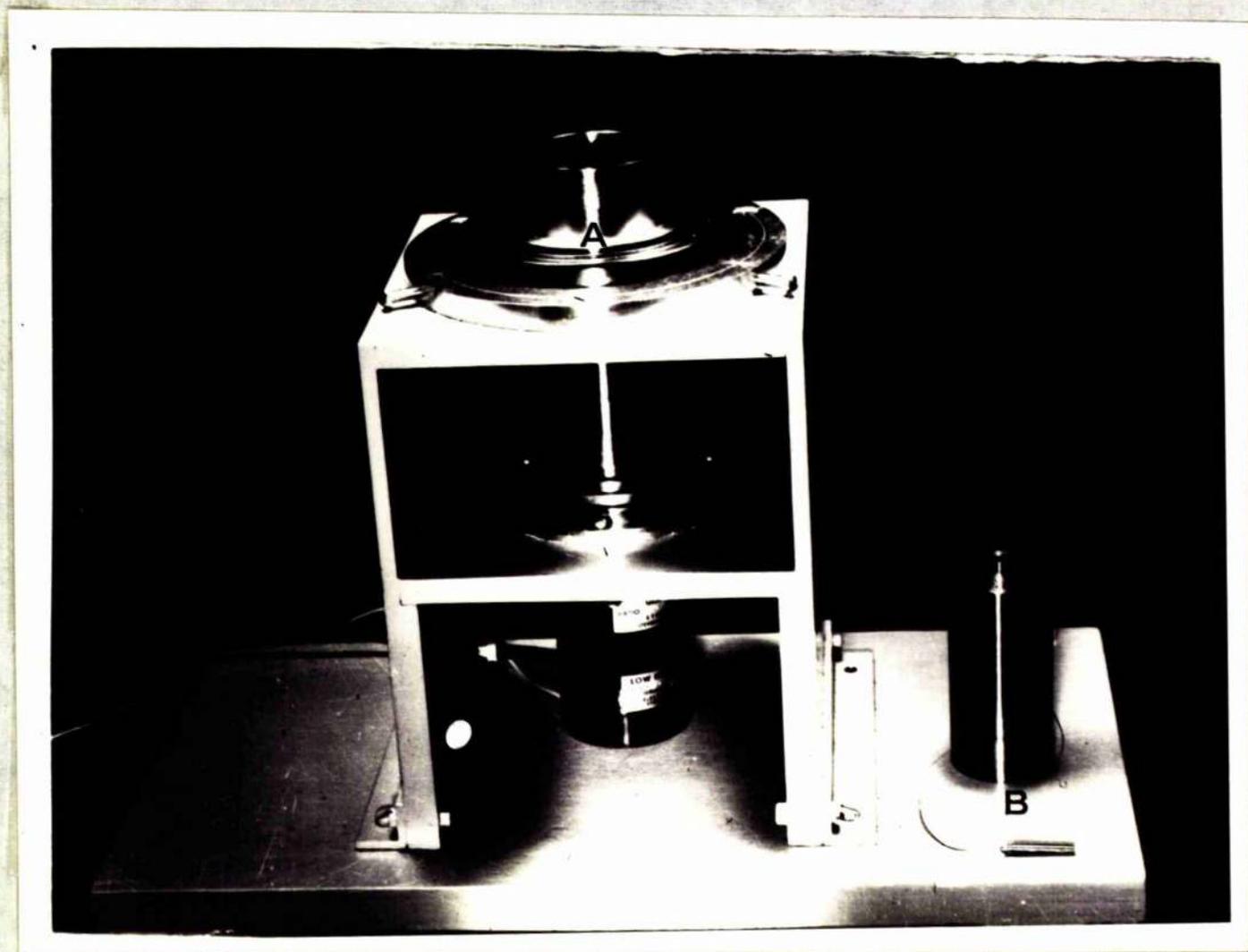


Fig. 1 General view of the Couette viscometer.

A, outer cylinder B, inner cylinder or bob.

$$G = W \frac{(R_o^2 + R_i^2)}{(R_o^2 - R_i^2)} \quad (7)$$

where W is the angular velocity of the rotating cylinder, and R_o and R_i 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 aggregates and will tend to break up aggregates. Thus for any given energy of adhesion (value of collision efficiency), at a given shear rate, G , there will be an equilibrium size for aggregates at which the rate of aggregation is equal to the rate of break-up of aggregates. The higher the shear rate, the smaller will be the maximum size of the aggregates and the sooner will equilibrium conditions be attained. Since the kinetics of the aggregation depart significantly from those given by equation (3), as the equilibrium condition is reached, it is important that measurements are made before this point. Knowledge of the shear rate also allows an evaluation of the contribution of Brownian motion in effecting collisions./

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

$$J/I = \eta \frac{(r_1 + r_2)^3 G}{2kT} \quad (8)$$

where kT has the usual meaning. For the lowest shear rate used, 1 sec^{-1} , and with particles of radius 5μ 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 viscometer used was constructed by Mr. E. German of the Department of Engineering, University College London. The cylinders are made of EN58B stainless steel, which is resistant to corrosion by saline solutions and the surfaces are machined to a surface finish of 10μ . The inner cylinder is freely suspended on a torsion wire while the outer cylinder is rotated to give a constant rate of shear by means of an integrating motor/

motor (Ether Ltd., Stevenage, Herts.) which runs at a very stable speed. The speed of rotation of the outer cylinder can be varied by changes of voltage or of gearbox, to give values from 1 revolution/second to 1 rev./min. The radii of the cylinders are 19mm and 20.5mm and thus shear rates in the range 1 sec^{-1} to 80 sec^{-1} are attainable and these fulfil the conditions discussed above.

The viscometer was set up some hours before use to allow it to reach the temperature at which measurements were to be made. The inner cylinder or bob was carefully centred inside the outer rotating cylinder. The axis of the outer cylinder was aligned vertically by careful levelling as any misalignment of the two cylinders leads to the bob rotating backwards and forwards and in even more complex modes, giving variable shear conditions.

(iii) Formation of Phospholipid Spherules

A small amount of a chloroform solution of the phospholipid was placed in a round-bottomed flask and the chloroform was evaporated in a rotary evaporator to leave a thin coating of the phospholipid in the flask. Appropriate salt solutions were added/

added and a dispersion prepared by shaking for 5 minutes with a vortex mixer (Fisons "Whirlimixer"). The suspensions were prepared so that a final particle (spherule) concentration of $1.0 - 1.5 \times 10^6$ per ml. was attained. Just before use the suspension was passed through two electroformed sieves (E.M.I. Electronics Ltd., Hayes, U.K.) one of sieve size 28 and the second 15. A sample was taken just before shear was applied and at frequent intervals subsequently, by means of a pipette, and the total particle concentration (single particles and aggregates) determined. These measurements were made using haemocytometers (modified Fuchs-Rosenthal) with Heine phase-contrast and Darkfield microscopy. The diameters of the spherules were measured at the same time, using an eyepiece micrometer. The spherules were also examined with a Polarisation Microscope and the Leitz Interference Microscope.

(4v) Solutions

All reagents were A.R. grade. The water used was double-distilled over glass and distilled a third time in an all silica still over K Mn O₄.

This/

This gave water with a specific conductivity of less than 1 micromho. cm^{-1} (Radiometer Conductivity meter). Solutions were made up using Grade A glassware. All glassware used was washed before use with the distilled water.

(v) Purification of Phospholipids

The method used was based on that given by Ansell and Hawthorne (1964). The yolks were separated from 6 eggs and homogenised with 250 mls. of Acetone at 20°C . The extract was filtered and the precipitate re-extracted as before. The precipitate was then shaken with 100 mls. of chloroform-methanol (1:1)v/v and filtered. The extraction was twice repeated and the extracts were combined. This method leads to small losses of phospholipids owing to their slight solubility in Acetone (see Ansell and Hawthorne 1964). The phospholipid mixture resulting from this extraction was applied successively to Alumina and Silica columns to separate off the lecithins (Phosphatidyl choline). / Column chromatography on Aluminium Oxide.

Aluminium Oxide ("Camag" Brockmann activity 1, Hopkins and Williams Ltd.) in chloroform-methanol (1:1, v/v) was packed in a glass column (1.5 cm diameter x 30 cm) and the column was washed with 100 ml/

ml of the same solvent. Approximately 1 gram of the above crude egg phospholipid was loaded in the column in chloroform-methanol (1:1, v/v) and eluted under pressure using a peristaltic pump (LKB) with the same solvent. Fractions were collected on an LKB fraction collector./ Column chromatography on silicic acid.

Silicic acid (Sigma Lipid chromatography grade) was heated at 110°C overnight and packed in a glass column (1.5 cm x 30 cm) in chloroform and washed with 100 ml. of the same solvent. The fraction from the alumina column (0.5 gms) was loaded in chloroform. Elution was carried out with chloroform-methanol (7:3 v/v) and the fractions collected on an L.K.B. fraction collector.

Samples were stored in chloroform under nitrogen at -20°C. Phosphatidyl serine obtained from Koch-Light, prepared by the method of Folch (1948), was also used. This was further purified on a silicic acid column prepared as above. The lipid was applied in chloroform-methanol (95:5, v/v), the column was washed with 100 ml. of the same solvent and the phosphatidyl serine was eluted with chloroform-methanol (1:1, v/v).

(vi)/

(vi) Characterization of Fractions

Phospholipids were determined by means of total phosphorus determination carried out on each fraction from the columns. The method used was a modification of that of Lowry et al (1954) (Dr. A. J. Lawrence pers. comm.) 0.25 mls of each fraction was placed in a stoppered tube and the solvent evaporated off. 0.25 mls of 10 M perchloric acid was added and the tubes kept at 170°C for 2 hours. The phosphate in the digests was determined colorimetrically. To each tube was added 0.5 mls. of a solution made up with 95 mls of 0.25% Ammonium Molybdate solution and 5 mls. of 0.25% Ammonium Meta vanadate solution in 0.1 N sodium Acetate to which 1 gram of ascorbic acid was added just before use. The tubes were incubated at 37°C for 30 mins. The optical density of the blue colour was determined at 660m μ on a Spectronic 20 colorimeter.

Once fractions containing phospholipids were determined, the phospholipids were characterised by thin layer chromatography, using a modification of the method of Parker and Peterson (1965).

Silica/

Silica Gel H (Merck A.G.), which contains no binder, was washed with methanol-chloroform-acetic acid (2:1:1, v/v/v) and glass distilled water. This was then dried at 110°C for 48 hours. 30 grams of the washed gel was mixed with 58 mls of glass distilled water in an homogenizer (M.S.E.). This was then used to coat glass plates, 20 cm x 20 cm, with a "Camag" applicator to a thickness of 300 μ m. The plates were air-dried and activated for 1 hour at 110°C just before use. Samples were applied as small spots (10 μ) using a microsyringe (Hamilton). Ascending chromatography with chloroform:methanol:acetic acid:water (25:15:4:2, v/v/v/v) was used. Plates were developed in a "Camag" "sandwich" chamber which is saturated with the vapours of the solvent mixture. The plates were air-dried and spots located using Iodine vapour. The spots were marked and sprayed with ninhydrin (BDH) and heated for 5 mins. at 110°C to identify free amino groups. Phosphate groups were identified by a molybdenum reagent (Dittmer and Lester's (1964) modification of the Zinzadre reagent), (see photograph fig.2).

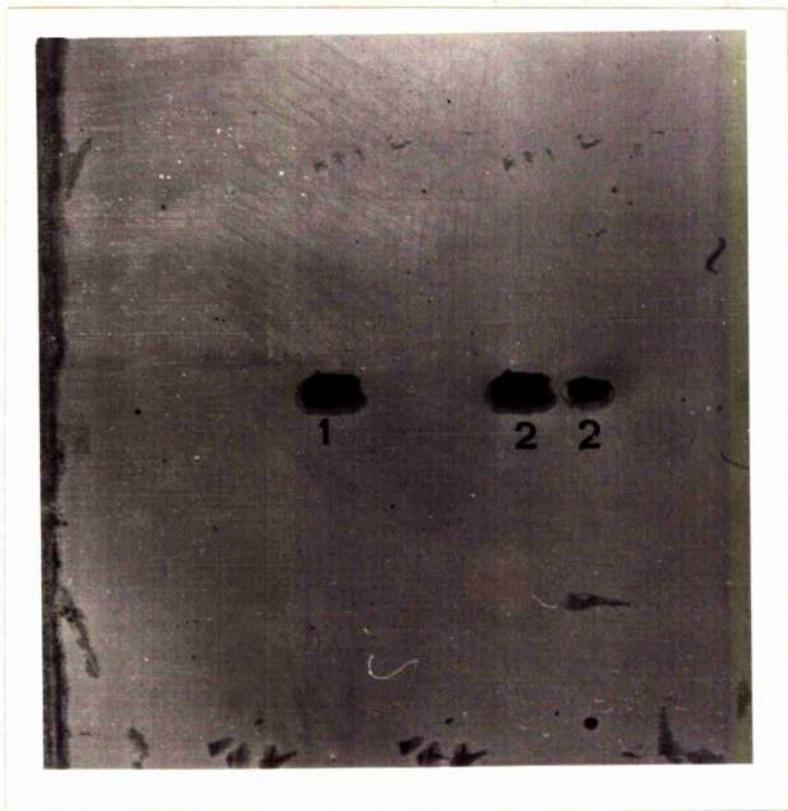


Fig. 2 T.L.C. plate stained with Phosphomolybdate reagent

See text for details.

1, Pure synthetic Lecithin (Mann) 2, Samples of purified
egg yolk lecithin

RESULTSCharacterisation of Materials

Egg phospholipid was eluted from the alumina column in 2 ml fractions and was found in the fifth and sixth fractions as determined by the presence of phosphate after ashing. The yield from this column was 60 - 70%. The combined fractions were applied to the silicic acid column and 2 ml fractions were again collected and the peak of the phosphate was found in fractions 10-12. Up to about 80% of the applied material was collected. Samples eluted from the two columns were run on thin layer plates and compared with samples of pure synthetic phospholipids. The crude phosphatidyl choline eluted from the alumina column showed at least two distinct spots. That from the silicic acid column developed only one spot which was ninhydrin negative and phosphorus positive. (See photograph Fig.2).

Phosphatidyl serine was eluted from the column as above and also run on a thin layer plate and gave one ninhydrin and phosphorus positive spot. The R_F values were similar to those of the synthetic compounds.

Microscopical/

Microscopical Examination of Spherules

When the dispersions were examined by means of phase contrast and dark field light microscopy, the lecithin particles were found to range from the approximately spherical to prolate spheroid in shape. Very few tubular myelinic figures ($< 2\%$) were observed after the dispersions were passed through the sieves. The radius of the spherules (particles) was measured and found to reasonably constant with a value of $5 \mu \pm 0.8 \mu$ S.E., independent of ionic conditions.

When the lecithin spherules were examined by means of a polarising microscope, they exhibited birefringence. The sign of the birefringence was positive with the refractive index for the radial direction being greater than that for the tangential (Frey-Wyssling 1953). The birefringence for such particles is the sum of two components. The intrinsic birefringence results from the alignment of individual phospholipid molecules in the lamellae and is usually positive. The form birefringence is a consequence of the fact that the spherules are made up of alternate shells of phospholipid and water. Such stacked/

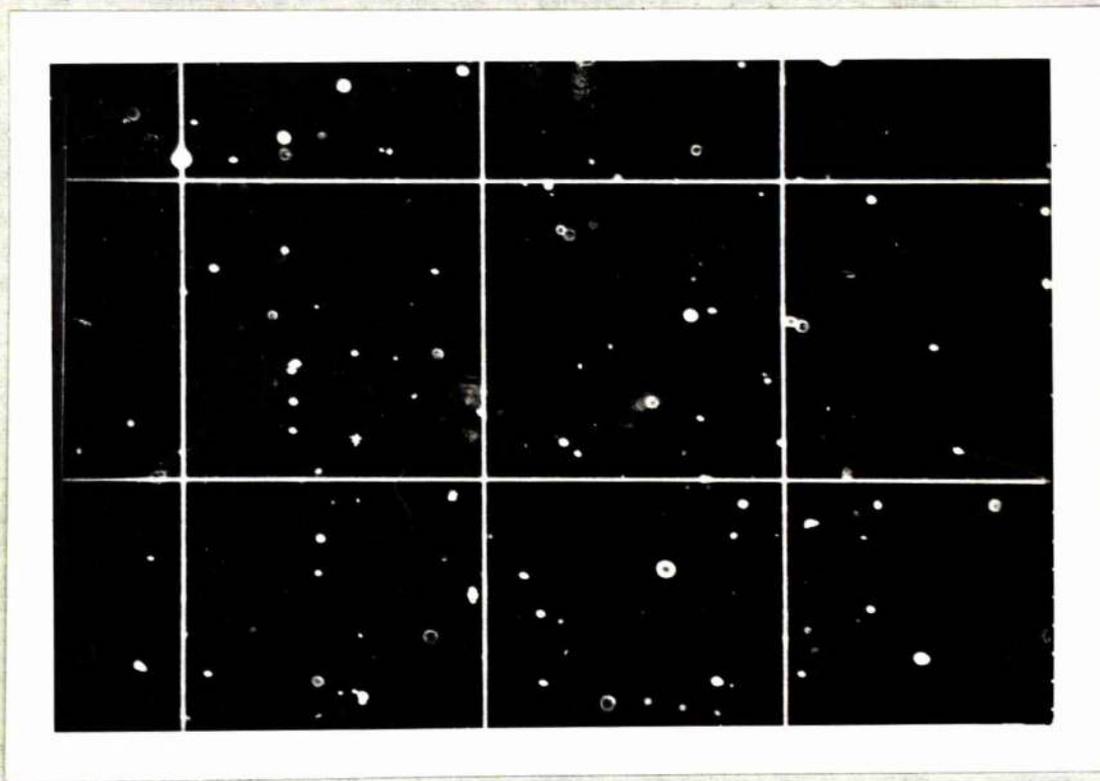


Fig. 3 Spherules of lecithin after 5 minutes aggregation under dark field illumination on a Fuchs-Rosenthal haemocytometer. Medium 1×10^{-3} M. CaCl_2 Marker Side of square 250μ

stacked layers lead to negative birefringence which is generally weaker than the intrinsic component. The fact that the sum of these two components, in this case, give strong positive birefringence indicates that the form birefringence is relatively weak and suggests a close spacing of the lamellae of phosphatidyl choline. The sign and magnitude of the birefringence did not change significantly with a change in ionic conditions (see discussion).

Phosphatidyl serine spherules exhibited a weak positive birefringence which decreased as the ionic strength of the suspending solution was increased and with higher valency cations. This indicates an increase in the form component and hence, probably, an increased inter lamellar spacing (Frey-Wyssling 1953, Papahadjopoulos and Miller 1967).

Interference microscopy confirmed the results obtained with the phase contrast microscope and examination of suspensions by means of interference contrast fields showed that after filtering the spherules were of similar size, i.e. monodisperse suspensions.

Results of aggregation in Couette Viscometer with lecithin spherules

1) Under/

1) Under different shear rates

The effect of different shear rates on the aggregation and hence upon the collision efficiency was studied using suspensions of lecithin spherules. Suspensions in 0.1M, 0.01M and 0.001M sodium chloride and calcium chloride were used. The results are given in table . In all the aggregations, the plot of $\ln N_{\infty t}$ against t gave a straight line relationship indicating that the aggregations followed the kinetics given above (equation 3), i.e. first order kinetics. The values for the collision efficiency given in table I are all based upon at least 5 experiments with six measurements of the total particle number at ten minute intervals. A typical example of such an experiment is given in table and the values plotted in figure 3 as $\ln N$ against t . Equation 6, above, indicates a dependence of the collision efficiency on the shear rate. The form of this dependence is given by the relationship between the interaction parameter K and the collision efficiency. Curtis and Hocking (in press) derived such a relationship indicating that the dependence was of a logarithmic form (see below). Thus from equation 6, a plot of $\log G$ against α , the collision efficiency, /

Table I : Effect of Shear Rate on Collision Efficiency

Temperature : 20°C. Lecithin dispersions n = 5 experiments

Shear Rate G in sec. ⁻¹ (log. G)	Collision Efficiency as % ± S. E.					
	1 × 10 ⁻¹ M NaCl	1 × 10 ⁻² M NaCl	1 × 10 ⁻³ M NaCl	1 × 10 ⁻¹ M ¹ CaCl ₂	1 × 10 ⁻³ M CaCl ₂	1 × 10 ⁻⁶ M CaCl ₂
1.98 (0.297)	27.5 + 0.78 -	16.5 + 0.89 -	10.2 + 0.58 -	23 + ± 0.64 -	30.1 + 0.85 -	15.4 + 0.53 -
5.15 (0.712)	24.1 + 0.91 -	11.1 + 0.38 -	7.1 + 0.25 -	19.5 + 0.77 -	26.4 + 0.59 -	11.5 + 0.8 -
8.35 (0.922)	22.8 + 0.56 -	10.8 + 0.67 -	5.5 + 0.84 -	17.6 + 0.38 -	24.5 + 0.91 -	7.3 + 0.66 -
13.911 (1.143)	19.8 + 0.67 -	8 + 0.54 -	3.6 + 0.33 -	15 + 0.39 -	22 + 0.69 -	7.3 + 0.28 -
41.73 (1.62)	15.5 + 0.34 -	6 + 0.56 -	2.8 + 0.74 -	9.1 + 0.21 -	16.3 + 0.42 -	5 + 0.19 -
64.2 (1.808)	14.4 + 0.22 -	4 + 0.36 -	1.4 + 0.17 -	7 + 0.32 -	12 + 0.48 -	3.6 + 0.25 -

Table II : Decrease of Particle Number $N_{0,t}$ in Typical ExperimentTemperature : 20°C. Medium 1 × 10⁻³ M CaCl G = 13.91 sec.⁻¹

Time t in minutes	Particle No. $N_{0,t} \times 10^{-5}$	$\ln(N_{0,t} \times 10^{-5})$	Collision Efficiency α as %
0	14	2.64	-
10	6.16	1.818	22.2
20	2.4	0.875	23.6
30	1.52	0.419	20
40	0.432	-0.839	23.5
50	0.22	-1.514	22.2
60	0.16	-1.833	20.5
* 70	0.136	-1.995	17.9

* Omitted from calculations, see Results section.

Fig. 2 Plot of N_{∞} and $\ln N_{\infty}$ against time

(Results from Table II)

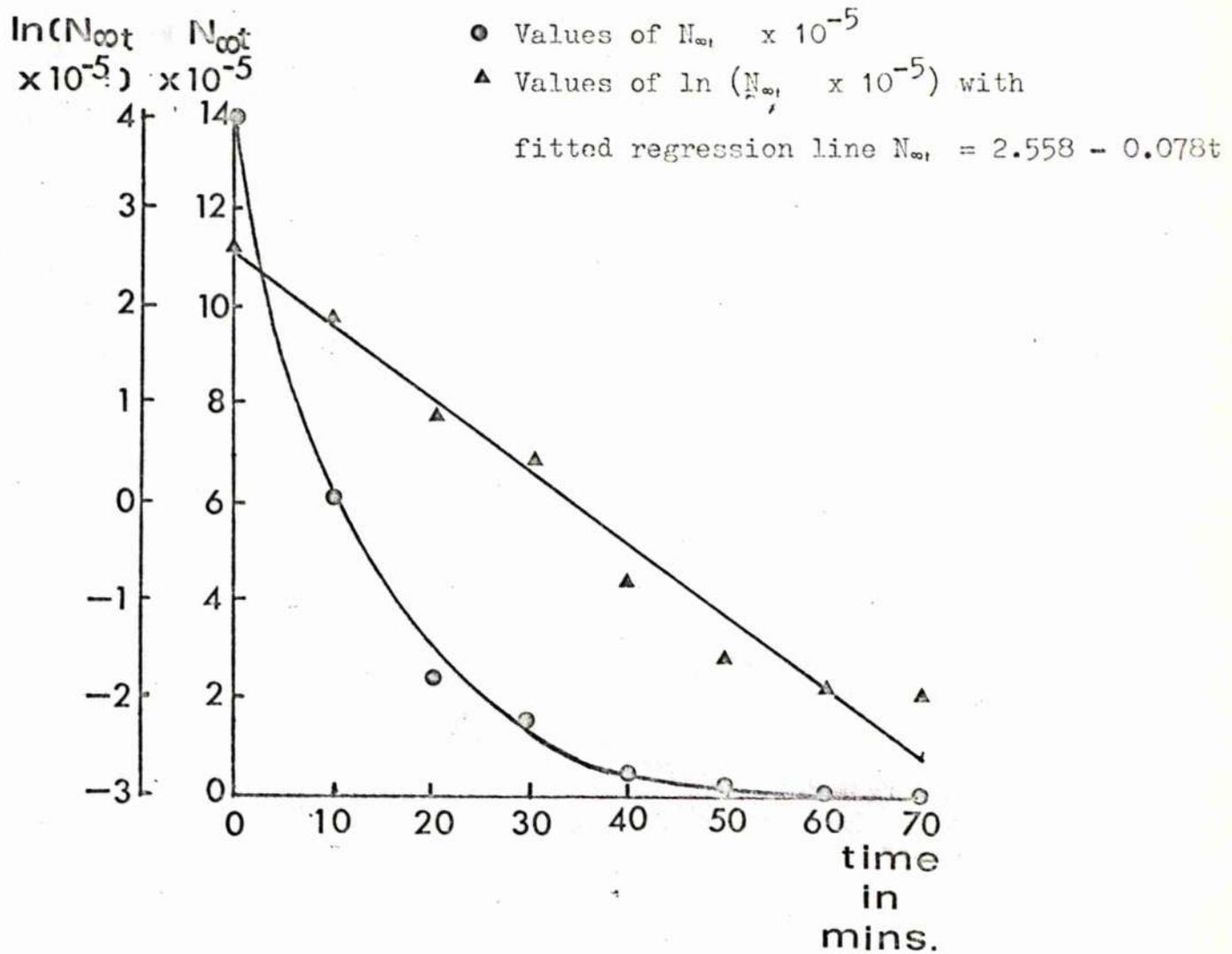
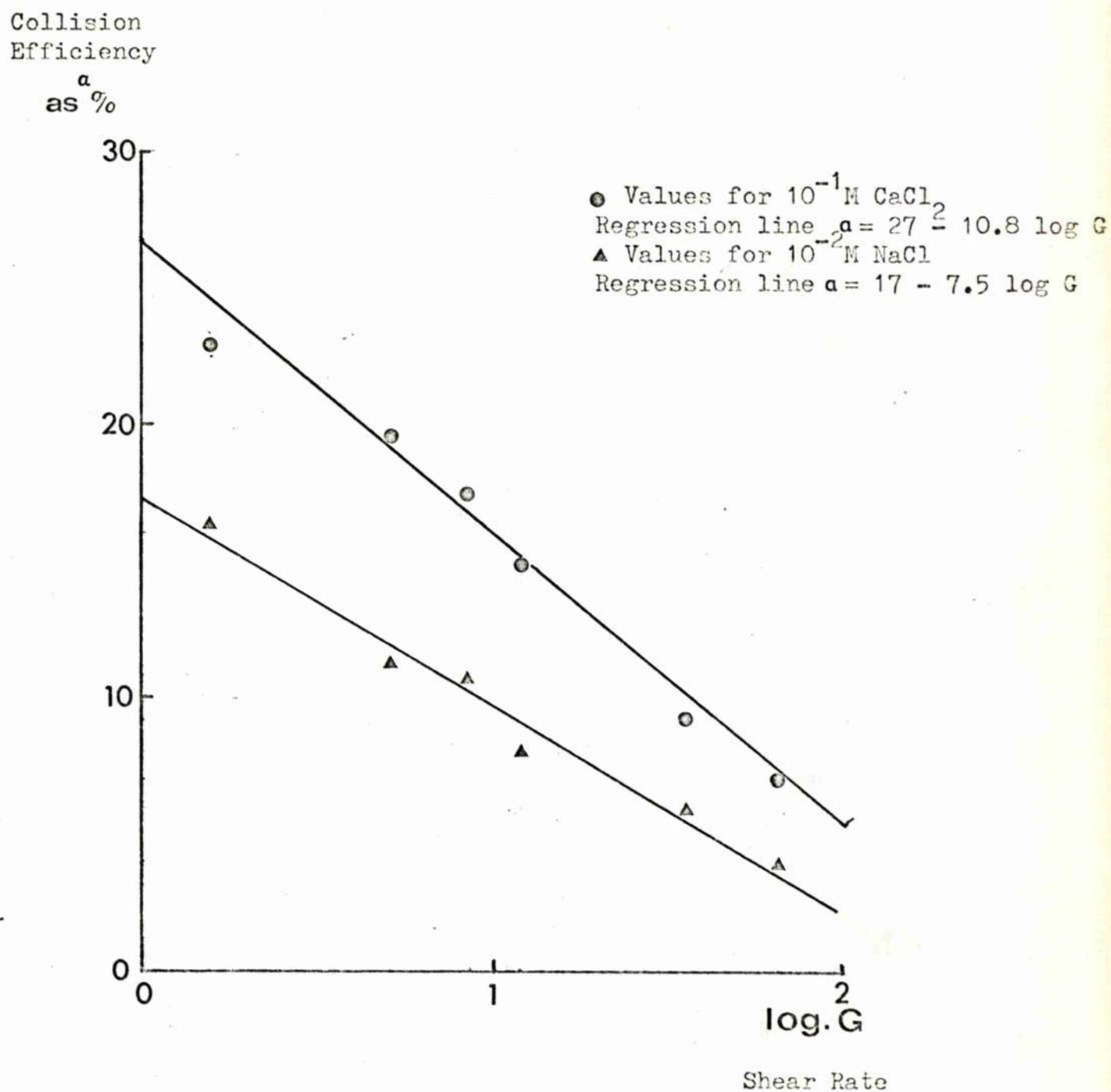


Fig. 3 : Sample Plots of Collision Efficiency against Log. Shear Rate
(Results from Table I)



efficiency should give a straight line relationship. This was found to be the case for the suspensions of lecithin under the conditions studied (see table and figure). This confirms the application of the treatment of Curtis and Hocking to these aggregations. When measurements were extended beyond 1 hr. the measured value of the stability ratio fell, indicating a departure from the kinetics of equation 3, possibly because of approach to equilibrium conditions. With suitable ionic conditions (see below) visible aggregates were obtained if the aggregation was continued overnight.

ii) Under different ionic conditions

Measurements of the collision efficiency for different ionic conditions but with a constant value for the shear rate are given in Table . As before, all aggregations were found to obey the kinetics of equation 3. From the values of the collision efficiency the London Hamaker constant, A , for $10^{-3} \text{M} \text{CaCl}_2$ was calculated using a value for the dispersion frequency of (see below), by means of equation 6, which is also tabulated in Table .

A problem in studying the effect of changing the/

Table III : Effect of Ionic Conditions on Collision Efficiency

Temperature : 20°C. G = 13.91 sec. n = 6 experiments

Conc ⁿ . of medium.	Collision Efficiency α as % \pm S. E.							
	LiCl	NaCl	KCl	MgCl ₂	CaCl ₂	SrCl ₂	BaCl ₂	Na ₂ SO ₄
1 x 10 ⁻¹ M	20.7 0.45	19.8 0.58	17.5 0.74	8 0.6	15 0.56	10 0.32	12.1 0.46	18.2 0.56
1 x 10 ⁻² M	11.6 0.32	9.1 0.58	7.3 0.74	<<1	<<1	<<1	<<1	8 0.44
1 x 10 ⁻³ M	5.4 0.12	4 0.31	2.9 0.17	16 0.58	22 0.66	19.1 0.22	20.2 0.73	4 0.26
1 x 10 ⁻⁴ M	2.1 0.22	1.5 0.31	1.02 0.19	12.1 0.45	16.4 0.53	13.3 0.47	14.2 0.39	1.2 0.22
1 x 10 ⁻⁵ M	<<1	<<1	<<1	8 0.33	10.7 0.52	9 0.41	9.8 0.48	<<1
1 x 10 ⁻⁶ M	<<1	<<1	<<1	5.3 0.46	7.5 0.33	6.2 0.47	6.6 0.38	<<1

Ionic Concentration of LaCl ₃	1 x 10 ⁻¹ M	1 x 10 ⁻² M	1 x 10 ⁻³ M	1 x 10 ⁻⁴ M	1 x 10 ⁻⁵ M	1 x 10 ⁻⁶ M
Collision Efficiency	13.1	6.5	1.3	<<1	20.3	15.2
α as % + S. E.	\pm 0.65	\pm 0.59	\pm 0.22		\pm 0.68	\pm 0.54

Dispersions of Phosphatidylcholine (lecithin).

the ionic conditions is the effect that any fortuitous change in the pH may have on the rate of aggregation. Measurements made at various times on the suspensions gave values for the pH which fell in the range 6-8, which variation would not be expected to be of significance (see below). Further experiments carried out using low concentrations of phosphate buffer (pH 7.2, ionic strength 0.0001 I) in the suspensions, gave values for the collision efficiency which were not significantly different from those measured on suspensions without a buffer.

The results are dealt with in detail in the discussion section.

iii) The effect of temperature

The above described experiments were carried out at 20°C. Similar experiments were also performed in a constant temperature room at 37°C. For these experiments, the lecithin was swollen in the test solutions at 37°C. Aggregations were also carried out at 2°C. The results for the three temperatures are compared in Table . The appearance of the lecithin spherules under phase contrast and polarisation microscopy at 37°C was similar to that at 20°C (radius $5.6 \pm 0.9 \mu$). At 2°C, spherules could be formed and their appearance under phase contrast microscopy, was also/

Table IV : Effect of Temperature on Collision Efficiency

$G = 13.91 \text{ sec.}^{-1}$ $n = 5$ experiments Lecithin dispersions

Temperat. in °C.	Collision Efficiency α as % \pm S. E.				
	1×10^{-1} M NaCl	1×10^{-2} M NaCl	1×10^{-1} M CaCl ₂	1×10^{-2} M CaCl ₂	1×10^{-6} M CaCl ₂
2°	18.6 \pm 0.45 $\bar{-}$	3.3 \pm 0.56 $\bar{-}$	14.3 \pm 0.62 $\bar{-}$	21 \pm 0.67 $\bar{-}$	6.2 \pm 0.55 $\bar{-}$
20°	19.8 \pm 0.67 $\bar{-}$	3.6 \pm 0.33 $\bar{-}$	15.0 \pm 0.39 $\bar{-}$	22 \pm 0.69 $\bar{-}$	7.3 \pm 0.28 $\bar{-}$
37°	20.2 \pm 0.58 $\bar{-}$	4.2 \pm 0.47 $\bar{-}$	16.5 \pm 0.59 $\bar{-}$	23.2 \pm 0.63 $\bar{-}$	8.1 \pm 0.48 $\bar{-}$

Table V : Aggregation of Phosphatidylserine Dispersions

Temperature : 20°C. $G = 13.91 \text{ sec.}^{-1}$ $n = 5$ experiments

Suspending Medium	Collision Efficiency α as % \pm S. E.					
	10^{-1} M	10^{-2} M	10^{-3} M	10^{-4} M	10^{-5} M	10^{-6} M
NaCl	16.2 \pm 0.68	6.8 \pm 0.72	2.1 \pm 0.37	1.0 \pm 0.1	$\ll 1$	$\ll 1$
CaCl ₂	21.3 \pm 0.71	16.0 \pm 0.55	10.0 \pm 0.38	6.4 \pm 0.43	2.6 \pm 0.21	1 \pm 0.11

Table VI : Viscosity of Suspensions

Measured with Ostwald Viscometer using η for Water at 20°C =
as Standard (Handbook of Chemistry and Physics 1966)

Conc ⁿ of Medium	Viscosity at 20°C. in Centipoises.							
	NaCl	LiCl	KCl	MgCl ₂	CaCl ₂	SrCl ₂	BaCl ₂	Na ₂ SO ₄
1 x 10 ⁻¹ M	1.00	1.60	1.001	1.006	1.008	1.008	1.011	1.001
1 x 10 ⁻² M	1.001	1.00	1.00	1.00	1.00	1.00	1.0	1.0
1 x 10 ⁻³ M	"	"	"	"	"	"	"	"
1 x 10 ⁻⁴ M	"	"	"	"	"	"	"	"
1 x 10 ⁻⁵ M				Ditto				
1 x 10 ⁻⁶ M				Ditto				

Temp. in °C.	Viscosity in Centipoises				
	1x10 ⁻¹ M NaCl	1x10 ⁻³ M NaCl	1x10 ⁻¹ M CaCl ₂	1x10 ⁻³ M CaCl ₂	1x10 ⁻⁶ M CaCl ₂
2°	1.7	1.69	1.71	1.69	1.67
20°	1.0	1.0	1.008	1.0	1.0
37°	0.71	0.69	0.72	0.69	0.69

also similar to that at 20°C (radius $5.9 \pm 1.0 \mu$). Spherules formed at 2°C were also examined with the polarisation microscope. Again the spherules were found to have strong positive birefringence.

iv) Aggregations with phosphatidyl serine

Spherules of phosphatidyl serine were formed at 20°C in $10^{-3}M CaCl_2$, $10^{-1}M CaCl_2$, $10^{-3}M NaCl$ and $10^{-1}M NaCl$. The results of these aggregations are given in Table . The calculated value for the London-Hamaker constant is also given in this table. Again a value of for the dispersion frequency was used. The suspensions were examined by phase contrast microscopy and the radius of the particles was measured as $5.1 \pm 0.7 \mu$.

Table VII : Value of London-Hamaker Constant A

Values of K and A calculated from equations 3 and 6

Medium & Dispersed Compound	Temperature in ° C.	α as % \pm S.E.	Interaction Parameter K	A in ergs
(1) Lecithin 1 x				
10^{-1} M NaCl	20°	19.8	2.6×10^{-6}	1.02×10^{-14}
10^{-3} M CaCl_2	20°	22	5.6×10^{-6}	2.2×10^{-14}
10^{-6} M CaCl_2	20°	7.55	2.5×10^{-9}	0.98×10^{-17}
10^{-3} M MgCl_2	20°	16	7.9×10^{-7}	3.1×10^{-15}
10^{-3} M SrCl_2	20°	19.1	1.99×10^{-6}	7.8×10^{-15}
10^{-3} M BaCl_2	20°	20.2	3.16×10^{-6}	1.2×10^{-14}
10^{-3} M CaCl_2	2°	20	2.818×10^{-6}	1.8×10^{-14}
10^{-3} M CaCl_2	37°	23.2	7.5×10^{-6}	2.45×10^{-14}
Phosphatidyl -serine.				
10^{-1} M NaCl	20°	16.2	1.12×10^{-6}	4.7×10^{-15}
10^{-1} M CaCl_2	20°	21.3	3.98×10^{-6}	1.66×10^{-14}
10^{-3} M CaCl_2	20°	10	6.31×10^{-8}	2.6×10^{-16}

Table VIII : Adhesive Energy of Particles

V_{att} . calculated from equation 5 using separation (H) of 5 A

Medium & Dispersed Compound	Particle Radius a in μm	London - Hamaker Constant	Attractive Energy V_{att} in ergs.	Attractive Energy in kT
1 x				
10^{-1} M NaCl Lecithin	5.0	1.0×10^{-14}	0.8×10^{-11}	210
10^{-3} M $CaCl_2$ Lecithin	5.0	2.2×10^{-14}	1.8×10^{-11}	453
10^{-1} M NaCl P.serine	5.1	4.7×10^{-15}	3.9×10^{-12}	99
10^{-3} M $CaCl_2$ P.serine	5.1	1.6×10^{-14}	1.4×10^{-11}	348

DISCUSSION

Any interpretation of experimental results relies on making assumptions about the test system, which of necessity limit the rigour with which the results can be applied. In the work detailed here, there are a number of assumptions involved, those resulting from the error of experimental measurements and those assumptions inherent in the theoretical treatment used. In any work involving the measurement of the physico-chemical parameters of a compound, an obvious basic assumption is the purity of the materials used. This was not easy to check in the case of the phospholipids. The major such check used was the technique of thin layer chromatography. This is considered to be one of the most reproducible and accurate methods available (Rouser et al 1968). The results of the T.L.C. analyses would seem to indicate that the phospholipids used were relatively pure although presumably consisting of a family of related compounds with differing hydrocarbon chains. However, the possibility of contamination by small quantities (say less than 1%) of/

of different phospholipids cannot be ruled out as such minor constituents may not show up with T.L.C. On the question of the purity of the inorganic salts used, one is, of course, in the hands of the manufacturers. There is no reason to believe that they are not trustworthy and further, all salts used were first roasted to get rid of any organic contaminants. The conductivities of the solutions compared very well with the values given in "The Handbook of Chemistry and Physics" (1966). This was used to check the concentration of the solutions used in these aggregations. Other sources of experimental error are dealt with in the results section.

The major assumptions of the theoretical treatment are (i) that the particles are spheroidal, (ii) that the suspensions are monodisperse, (iii) that the particles are electrically neutral so that no appreciable electrostatic potential energy barrier exists, and (iv) that the particles have a density which is inappreciably different from that of the suspending medium, i.e. the particles do not sink. Optical microscopy provides information on the validity of all these assumptions. Examination of the/
the/

the spherules by means of phase contrast and interference microscopy showed that a sufficient proportion of the spherules were spheroidal, so that the first assumption is probably not unreasonable. The second assumption that the suspensions consist of single particles of the same size can be borne out by the low standard errors of the particle radius and by the examination with interference contrast under which large deviations in particle size show up as differently coloured particles.

The third assumption really requires particle electrophoresis as its test. However, this was not possible and indications on the surface potential can in fact be deduced from the birefringence studies. As was stated in the 'Results' section the birefringence examination of lecithin spherules tends to indicate (Frey - Wyssling 1953) that the lamellae which are considered to constitute these structures are closely opposed. This fact and the independence of optical retardation with respect to ionic strength indicate that the surfaces of the lamellae and hence of the spherules are effectively electrically neutral or at least have a very low surface potential (see later discussion) and thus have a low electrostatic/

static force of repulsion to prevent the approach of two such surfaces. However, the results of birefringence studies on spherules of phosphatidyl ethanolamine tend to indicate an appreciable surface potential. The problems that this introduces into the treatment are dealt with below. These birefringence results are similar to those obtained by Papadjopoulos and Miller (1967) for the same compounds which tends to confirm the T.L.C. identifications.

The fourth assumption was simply tested by allowing a suspension to settle in the counting chamber of haemocytometer slide. It was found to take 30 minutes for the suspension to fully settle hence it took the particles 30 minutes to fall a maximum distance of 0.2mm (the depth of the chamber). Thus over the duration of the experiments, 1 hour, the particles would be expected to sink through a distance of some 500 μ which is 100 times their radius and about 0.5% of the depth of the Couette viscometer. This is too small a distance to affect the calculations.

Thus it would seem that the major assumptions made in applying the theoretical treatment to the experimental results are reasonable assumptions for this/

this system. However, this does not guarantee that the treatment is in fact appropriate for the system, and an evaluation of the relevance of the theoretical approach requires that the results be shown to be consistent with the theory. The results showed that in all experiments plots of $\ln N_{\infty}t$ against time were linear (this is further borne out by the low values of the standard errors of the collision efficiency) which shows that the aggregations followed the kinetics predicted by equation 3, confirming the use of this equation for this system. The dependence of the collision efficiency on shear rate is evidence that the theory outlined for the calculation of a force constant is suitable for these aggregations.

The values determined, from these aggregations, for the collision efficiency can be used in two ways; as a means of calculating an absolute value for the force constant and as a way of comparing the rates of aggregation when these are carried out under similar conditions. If the conditions, i.e. viscosity, shear rate $\dot{\gamma}$ and size of particle, are similar, it is obvious from equation 6 that a comparison of collision efficiencies is equivalent to a comparison of force constants. Hence it is possible to compare the/

the interactions of the spherules under differing physico-chemical conditions without calculating the force constant in each instance.

Values for the collision efficiency under different ionic conditions using spherules of lecithin are given in Table . These aggregations were carried out at a constant shear rate of 13.91 sec^{-1} . Equation 6 also demonstrates that the viscosity of the suspensions and the radius of the particles must be equal before the collision efficiency can be used to compare the adhesiveness of the spherules.

Measurements were made of the viscosity of solutions and suspensions with an Ostwald viscometer using the value of the viscosity of water at 20°C given in 'The Handbook of Chemistry and Physics' (1966). The values are given in Table . This Table demonstrates that there are no significant differences in the viscosities of the solutions used (maximum difference 0.1 centipoise). The question of the radius of the spherules is particularly important as the force constant is directly proportional to the cube of radius (equation 6). Measurements of the radii under different ionic conditions (see Results) showed/

showed that these did not differ with this particular mode of preparation (range 1μ), which is not much greater than the experimental error. Considering the low variability of both of these factors, it is probably reasonable to use the collision efficiency to compare adhesiveness.

The values in Table demonstrate three overall factors in the aggregation of the suspensions and hence, it is presumed, in the adhesion of the spherules: (1) The greater the concentration of the solution, the higher the value of the collision efficiency (for deviations see below) indicating an increased rate of aggregation and a greater adhesive interaction; (2) the lowest concentration of a salt which will effect measurable aggregation depends upon the valency of the ions in the salt, for example, measurable aggregation in solutions of sodium chloride begins at concentrations of the order of 10^{-4} molar, whereas aggregation was observed in concentrations down to 10^{-6} molar with calcium chloride solutions; (3) it was the valency of the cation which was effective in decreasing the concentration required to effect aggregation (flocculation value) as a comparison the results/

results with calcium chloride and sodium sulphate exemplify.

A more detailed examination of the results reveals other phenomena. Firstly, with divalent cations, the rate of aggregation increases with increasing concentration, up to a certain concentration, then falls and finally increases again (see Results for calcium chloride). Secondly, although in general divalent cations are more effective in promoting aggregation than are monovalent cations, there are also differences in the effectiveness of specific cations. The effectiveness of monovalent cations decreases in the order Lithium, Sodium, Potassium, whereas that of the divalent cations decreases in the order Calcium, Barium, Strontium, Magnesium. The actual differences between the values of the collision efficiency are, it should be pointed out, quite small, particularly in the case of the divalent cations, although they are consistent differences. The results for Lanthanum are similar, with reversal of charge occurring at lower concentration.

These results are consistent with an interpretation of the aggregation and adhesion of the lecithin spherules, based on Derjaguin-Landau, Verwey-/

Verwey-Overbeek theory of the stability of lyophobic colloids. The effect of increasing cation concentration reflects the increasing concentration of counter ions in the electrical double layer. These lower the surface potential by neutralising the surface charge and therefore decrease the electrostatic force of repulsion. The fact that it is the cation that is significant in neutralising the charge (see point 3 above) indicates that the spherules probably have a net negative surface charge. However, this is almost certainly very small as aggregation occurred in 10^{-4} M NaCl and in concentrations down to 10^{-6} M Ca Cl_2 . On this theory, as the concentration of the counter ions (cations) is increased, a concentration will be reached where the number of ions adsorbed onto the surface of the spherules is sufficient to compensate for the charge borne by the spherules. Higher concentrations will tend to impart a positive charge to the spherules, (Kruyt 1952, 1949). This should be reflected in the aggregation of the spherules. This is shown to be so when the values of the collision efficiency for spherules in solutions of divalent cations are examined. Starting from/

from the lowest concentration, in the case of calcium chloride solutions, the value of the collision efficiency increases up to 10^{-3} Molar, at which concentration the lecithin spherules are presumably at or about the zero point of charge. Next comes a range of concentrations where as a result of the positive charge contributed by the adsorbed calcium ions, there is a significant electrostatic force of repulsion to prevent adhesion, as can be seen from the very low value of the collision efficiency in 10^{-2} Molar calcium chloride. Subsequently, an increase of the concentration will lead to adhesion and aggregation of the lecithin spherules once more, as a consequence of the increase in the concentration of the new counter ion, the chloride ion. This is again found to happen with the increased value of the collision efficiency in 10^{-1} molar calcium chloride. This reversal of charge phenomenon can also be invoked to explain the changes in the value of α obtained for the aggregation of lecithin spherules in solution of other divalent cations (see Table). The reversal of charge seems, from these result, to occur at different concentrations with the different cations and the order of increasing concentration/ and Lanthanum

concentration at which it occurs is the same as order of the decreasing effectiveness in aggregation, namely Calcium, Barium, Strontium, Magnesium. It is to be expected that an increase in the concentration of the monovalent ions would also lead to such a reversal of charge, however this could not be investigated as the increased concentration also increased the density such that the spherules were buoyant and aggregation could not be observed.

The polar group of phosphatidyl choline (lecithin) is a zwitterion consisting of a phosphate group and a quaternary ammonium ion. There is much evidence to show that both acid and base in this amphoteric head group are fully ionised and the compound is isoelectric over a wide pH range (Bangham and Dawson 1959, Bangham 1968, Hanai, Hayden and Taylor 1965). It is thought that the quaternary ammonium ion and the phosphate are coplanar (Hanai, Hayden and Taylor 1965) which leads to an internally compensated zwitterion (Anderson and Pethica 1956). Shah and Schulman (1965) have questioned this conclusion, but their arguments are based on the results of monolayer studies, the relevance of which to/

to dispersions are not clear. The results discussed above are consistent with the model of a fully internally compensated zwitterion which may possess a net, albeit low, negative surface potential circa -1 millivolt (Hanai, Haydon and Taylor 1965). The effect of specific ions is also interesting. The order of the decreasing effectiveness of univalent cations is the same as the order of the increasing ionic radius:- Li:Na:K, which is also the order of decreasing energy of polarisation (Davies and Rideal 1963). This tends to suggest that the differences in univalent cations are due to the properties of the cations themselves and not to any specific affinity of the phospholipid, which is in line with the body of evidence that phospholipids do not show a specific affinity for any univalent metal ions (Bangham 1968). On the other hand, the results for divalent cations are not so straightforward. The order of effectiveness and of the reversal of charge concentration do not reflect the order of increasing ionic radius and polarisability which is Mg:Ca:Sr:Ba. The position of calcium seems to reflect a greater affinity of this ion for lecithin which would be confirmed by the results of Shah and Schulman (1965,1967) on the interaction of calcium and lecithin.

The effect of temperature on the aggregation of lecithin spherules was also investigated. Equations 3, 4, 5 and 6 demonstrate that the only temperature dependent term in this treatment is the viscosity, η , of the suspensions. Thus, aggregations at different temperatures can be compared if due allowance is made for the change in viscosity. From equation 6, it can be seen that the force constant, A , is directly proportional to the viscosity, and thus to compare adhesiveness on the basis of collision efficiency, allowance must be made for small decreases in the value of collision efficiency for an increase in the viscosity. The viscosity of solutions at the temperatures used are given in Table VI. This change in viscosity, with a maximum of the order of 0.3 centipoises, would be expected to alter the value of the collision efficiency by some ± 0.5 when expressed as a percentage, if the adhesiveness of the particles remains constant. This is a small correction and comparisons can be made of the aggregations at different temperatures without calculating a force constant in each case.

Temperature may be of importance in the adhesion
of/

of the lecithin spherules because of possible phase changes in the phospholipid (Luzzati 1968, Chapman and Wallach 1968), such phase transitions having been proposed as a mediator of membrane interactions (Kavanau 1965). Further, it is possible that temperature changes may cause a change in the conformation of the polar head group (see above) which would alter the surface charge of the spherules and hence their net adhesive interaction.

Table IV illustrates the fact that temperature has little effect on the aggregation and adhesion of lecithin spherules. The differences measured, ^{which are not} in the value of the collision efficiency, α , is significant at the 5% level by means of a 't' test, of the order expected, purely because of differences in viscosity. This is confirmed by the birefringence results which tend to indicate that the spherules are similar at the three temperatures. This is not unexpected as the phase transition for egg yolk lecithin is thought to occur over a wide temperature range, probably covering the range of temperatures used, as it consists of lecithins with hydrocarbon chains differing in length and unsaturation, (Chapman and Wallach, 1968). Any other possible changes, /

changes, such as the orientation of the dipolar head group, are apparently not sufficient to alter significantly the adhesive interaction.

Phosphatidyl serine was used to provide information on the aggregation of spherules with a considerable negative charge. Phosphatidyl serine was chosen because it bears an extra negative charge at physiological pH, due to its carboxyl group. It was chosen in preference to phosphatidyl ethanolamine (cephalin) as the charge behaviour of this latter compound varies considerably with conditions of preparation and storage. Measurements indicate that phosphatidyl serine dispersions have a zeta potential (potential at the plane of shear) of some -60 millivolts (Bangham and Papahadjopoulos, 1966). The birefringence studies, as discussed earlier, indicated that the lamellae were charged.

The values measured for the collision efficiency are given in Table V. Because of the assumption, in the treatment of the collision efficiency, that the particles are uncharged there are obvious difficulties in the interpretation of these results. However, to use the collision efficiency as an approximate/

approximate guide to the aggregation and adhesion of the particles, the results of Table V illustrate the typical behaviour of a suspension of negatively charged particles (see earlier discussion on lecithin). In this case, there is no evidence for a reversal of charge as with the lecithin spherules and higher concentrations of any ion are required to effect flocculation of the spherules. Thus, detectable aggregation first appears in $10^{-3}M$ NaCl and in $10^{-5}M$ $CaCl_2$, which is to be expected from calculations using the formula given for the decrease of potential with distance from a charged surface. These show that if the diffuse double layer around the phosphatidyl serine spherules is treated as a flat double layer (which is quite reasonable for such large particles) the potential will have decreased exponentially to zero over a distance of the order of 50\AA in $10^{-2}M$ NaCl (Verwey and Overbeek 1948), thus allowing an adhesion in the secondary minimum (see Introduction). The fact that a fast aggregation occurs from $10^{-3}M$ $CaCl_2$ is in line with results on monolayers of phosphatidyl serine which indicated that there was a 1:1 binding of calcium ions to phosphatidyl serine molecules at about $10^{-3}M$ calcium

in/

in the presence of 0.145 M KCl/Tris-Cl (Bangham and Papahadjopoulos, 1966).

The problem of applying the treatment for uncharged particles to such spherules with a relatively high surface potential is solved by these two latter considerations. The values of collision efficiency measured in highest concentrations of cations, where the potential falls rapidly to zero, can be used in the theoretical treatment outlined above, as there is almost certainly no significant electrostatic force of repulsion at these concentrations (Verwey and Overbeek, 1948). The results of Bangham and Papahadjopoulos (1966) on the binding of calcium ions to phosphatidyl serine (see above) also indicate that at concentrations of the order $10^{-3}M$ calcium the charge on the spherules might be expected to be neutralised by bound calcium ions.

In a comparison of the results for phosphatidyl choline and phosphatidyl serine the structure of the polar head group and the surface potential are the two key parameters. The polar moiety of the two compounds differ in the group which is bound by an ester linkage to the phosphate group. As their names indicate in phosphatidyl choline the group/

group is the quaternary ammonium base, choline, with a structure $(\text{CH}_3)_3 \overset{+}{\text{N}}\text{CH}_2\text{CH}_2\text{OH}$ and in phosphatidyl serine it is the amino acid, serine, $\text{HOCH}_2\text{NH}_2\text{CH}_2\text{COOH}$. The phosphatidyl choline head group thus consists of the strong base choline and the moderately strong acid, phosphoric acid. These are ionised over a wide pH range and thus the head group is a zwitterion. Micro-electrophoresis (Bangham and Dawson 1959) shows that dispersions of this compound are iso-electric over a wide pH range. Hannai, Haydon and Taylor (1965) have argued that such a result leads to the conclusion that the choline and phosphate groups are co-planar in a plane perpendicular to the fatty acid chains of the molecule i.e. in the plane of surface of the particles (see also Pethica 1965). Shah and Schulman (1967) interpret their results from lecithin monolayers on the basis of the head groups being in the extended configuration with the quaternary ammonium ion closer to the plane of shear than the phosphate group. The sort of results presented here which indicate that the surface potential is low or zero are also in favour of the co-planar configuration rather than the latter configuration in/

in which the surface potential would be expected to be some 40mV positive. However, the high resolution proton magnetic resonance spectra of dispersions of egg yolk lecithin show a very marked peak for the protons of the choline group $N(CH_3)_3$, Chapman and Wallach, 1968, which would suggest a considerable amount of movement for this group. On the other hand, X-ray studies on L- α -glyceryl phosphorylcholine cadmium chloride trihydrate show that in the crystalline state the choline residue is in the 'gauche' conformation rather than the extended form (Sunderabingam 1968), which would tend to hinder free rotation.

Such considerations lead to a re-interpretation of the results of the aggregations for lecithin spherules. If the head group is in the co-planar configuration the surface potential would be expected to be zero (see above) and the reversal of charge phenomenon observed with divalent cations reflects the binding of calcium ions, presumably to the phosphate group. If the spherules have no charge, there should be no diffuse layer of ions round the spherules and the results should reflect changes in the stern layer (the layer of ions adsorbed/

adsorbed at the surface) and not changes in thickness of the diffuse layer. However, the results for monovalent cations, which show that a concentration of 10^{-4} M NaCl is required for aggregation to occur, would suggest that there is some charge on the spherules and the decrease of the flocculation concentration with increasing valency of the cation suggests that the net charge is negative. Similar results were obtained by Thomas (1962). This is somewhat difficult to reconcile with the standard representation of the head group. It is possible that the results reflect contaminants in too small a concentration to be detected by T.L.C., for example, phosphatidic acid. The results, however, clearly demonstrate that phosphatidyl choline binds divalent cations, in particular calcium, quite strongly, confirming the results of Shah and Schulman (1967 a & b). Presumably the calcium is bound to the phosphate group but the nature of the specific mechanism for this binding is not yet clear. The possibility of changes in the conformation of the dipolar head group cannot be ruled out and it is possible that binding of cations may favour a particular conformation and stabilize it. As temperature/

temperature seems to have no significant effect on the aggregations, it presumably does not significantly alter the head group conformation.

Phosphatidyl serine on the other hand has a negative surface potential, which is a consequence of the acidic serine group. This has an amino group with a pKa of about 9 and a carboxyl group with a pKa of about 2. Thus at physiological pH it has an extra negative charge compared to lecithin. Spherules of this compound will therefore be expected to have a diffuse double layer about them and increasing ionic strength should not only result in increased adsorption of counter-ions in a stern layer but also a decrease of the diffuse double layer thickness. Both of these effects will contribute to a diminution of the force of electrostatic repulsion. The results demonstrate the latter effect and a comparison of the minimum concentration of monovalent and divalent cations required to produce aggregation, namely $10^{-3}M$ NaCl and $10^{-5}M$ CaCl₂, show that they are in the ratio (100:1) which is to be expected if the effect is primarily on the thickness of the diffuse double layer (Kruyt 1952). The increased rate of aggregation, as reflected in an increased value/

value of the collision efficiency, with a further increase in the ionic strength may also result from specific adsorption of cations in the stern layer, which is to be expected from the results of Bangham and Papahadjopoulos (1966) which suggested that above 10^{-3} M CaCl_2 , there is a change in the binding complex, giving a higher calcium to phosphatidyl serine ratio. Unfortunately the results, in this case, allow no test of binding in the stern layer as no charge reversal was observed in the concentration range used although stern binding certainly occurs. The results obtained agreed with those of Abramson, Katzman and Gregor (1964).

The two compounds thus show binding of cations, probably to the phosphate group in the case of lecithin, while in phosphatidyl serine both the phosphate and the carboxyl group are available for the binding of cations. In both cases, divalent cations are bound more strongly than monovalent cations and lecithin shows a greater affinity, as shown by reversal of charge concentration and collision efficiency, for calcium ions than for the other divalent cations used. The order of this increasing/

increasing affinity Mg:Sr:Ba:Ca is different from that obtained by Bungenberg de Jong (Kruyt 1949), - Sr:B:Mg:Ca, but it seems likely that not very pure samples of egg lecithin were used in this latter study. The differences in the values of the collision efficiency which are used to adduce the differences in affinity are small but they are consistent differences. The results obtained for the two compounds do not allow the calculation of binding constants for the cations and only useful for qualitative comparisons. ^{are}

One of the main purposes of this study was to attempt a measurement of the London-Hamaker constant and the adhesive energy of these phospholipid spherules. The theory behind the measurement has been outlined in the "Material and Methods" section. As was discussed at the beginning of this section, the theory for evaluating the London-Hamaker constant from a measured value of the collision efficiency, is based on a number of assumptions which are demonstrably reasonable in the case of lecithin. The London-Hamaker constant, A , (see equations 4, 5 and 6) is a characteristic parameter of the interaction of agglomerations of molecules (Kruyt/

(Kruyt 1952). Thus its value in the interactions under different ionic conditions should be constant assuming that the ionic concentrations do not affect ϵ . The different values obtained for the collision efficiency reflect a change in the total interaction energy which is the sum of the London attractive energy and the energy of electrostatic repulsion, the latter changing with the ionic conditions but not the former. As outlined earlier, one of the major assumptions of this theory, is that there is no significant force of electrostatic repulsion. This assumption is reasonable in the case of lecithin spherules which have at most a very low surface charge. Thus it was decided to calculate the London-Hamaker constant from the collision efficiency obtained for aggregation under the most favourable conditions. These were the highest concentration of monovalent cations and the highest concentration of divalent cation achieved before charge reversal. The latter was chosen, namely $10^{-3}M$ calcium chloride as this is probably somewhere near the zero point of charge of the spherules and most closely fulfils the above assumption. The value calculated from the results at $20^{\circ}C$ for lecithin/

lecithin and phosphatidyl serine is given in Table VII. Values based on the experiments in $10^{-3}M$ calcium chloride at $2^{\circ}C$ and $37^{\circ}C$ are also listed in Table VII. A value of 2.8×10^{15} es/sec was used for the dispersion frequency (see below).

These values for the London-Hamaker constant can be compared with those derived from theoretical calculations and by other experimental methods. It is possible to derive a value for the constant, B , for the interaction of two isolated molecules which is given by

$$B = \frac{3}{4} h \nu_i \alpha^2_0 \quad - 9$$

(Meelwyn-Hughes 1961) where the energy of interaction is given by $-\frac{B}{r^6}$, with $h\nu_i$, the ionisation potential (ν_i is the frequency of vibration of the dispersion electron) and α_0 the polarisability of the molecule. Other methods for computing B , from the optical constants of the molecule, due to a number of authors are given in Meelwyn-Hughes 1961). They require a knowledge of the refractive index over a range of wavelengths. The value of A can be derived from equation (9) by means of the expression (Kruyt 1952),

$$A = \frac{2}{3} \frac{2B}{q} \quad - 10$$

where/

where q is the number of atoms per cm^3 . This expression is based on the assumption that the forces between molecules are strictly additive, which may not be true for the passage of the force through a dense medium. Lifshitz and 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 loss factor of the interacting materials. In terms of the energy of attraction between two semi-infinite regions of material "1" separated by a small planar gap, C , of medium "2", the expression is

$$E = - \frac{\hbar}{16\pi^2 C^2} \int_0^\infty \left[\frac{\epsilon_1(i\xi) - \epsilon_2(i\xi)}{\epsilon_1(i\xi) + \epsilon_2(i\xi)} \right] d\xi \quad (11)$$

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 media evaluated on the imaginary axis in the complex frequency plane, (Parsegian and Ninham 1969).

Although/

Although this latter method overcomes the difficulties of the pairwise summation method and allows for the medium intervening between the two interacting bodies, it requires a knowledge of the dielectric data over a wide frequency range, except for interactions at large distances ($> 1000 \text{ \AA}$) where the static dielectric constant can be used. As the interactions under consideration are probably considerably less than this (ca. 100 \AA , see below), it might seem that the Lifshitz theory is of no more use than the London-Hamaker equations. However, Parsegian and Ninham (1969) have calculated a theoretical constant for the force in thin lipid films using data for hydrocarbon films, with a number of assumptions which simplify the calculation of expression 11. The value of the London-Hamaker constant A , they calculate is 4.5 to 5.4×10^{-14} ergs.

Haydon and Taylor (1968) derived a value of A of 5.6×10^{-14} ergs from the contact angles for thin films of glycerol mono-oleate in *n*-decane. Other lipids have been studied, in particular arachidic acid soles (Ottewill and Wilkins 1962) which gave a value of 6×10^{-15} ergs. Although no other values are available for phospholipids (see however, Haydon 1967), it is not unreasonable to compare the values calculated/

calculated from the collision efficiency with the values listed above.

The values of A given in Table VII, were calculated using a value of the characteristic wavelength of $1.06 \times 10^{-7} \text{ m}$, based on the vibration frequency of 2.8×10^{15} cycles/sec. given by Parsegian and Ninham (1969) for ethane. Ottewill and Wilkins (1962) give a value of 2.73×10^{15} cycles per second for stearic acid. This is not too serious an assumption as the effect of retardation is probably small for these aggregations (see below). The values obtained for both lecithin and phosphatidyl serine are in the range $0.9 - 2.5 \times 10^{-14}$ ergs. This is within the range of values which have been obtained for other lipids (see above) and the values are thus consistent with other theoretical and experimental determinations.

From the values of the London-Hamaker constant, the energy of the attraction was calculated. To do this, a knowledge of the separation of the adhering particles is required. In the Introduction, it was pointed out that on the Derjaguin, Landau, Verwey, Overbeek theory of colloid stability, particles may adhere in the so-called primary or secondary minimum. The/

The first is characterized by strong adhesions which are not readily re-dispersed and the latter by weak, re-dispersable adhesions. The aggregates formed in 10^{-6} M divalent cations and in 10^{-3} M monovalent cations were readily re-dispersed mechanically and these probably represent adhesions in the secondary minimum. However, at 10^{-3} M divalent cations (10^{-1} M Ca for phosphatidyl serine) and 10^{-1} M monovalent cations very stable aggregates were formed. These were the concentrations for which the London-Hamaker constant was calculated and the energy of attraction has also been calculated for this value, assuming that the adhesions are in the primary minimum with a separation of 5\AA . It is theoretically possible to calculate the energy of adhesion in the secondary minimum but this requires more certain knowledge of the distance of separation, which can vary over the range $100-200\text{\AA}$, and also involves computational problems (Curtis and Hocking in press). With a separation of 5\AA , the retardation correction is negligible, as this separation is very small compared to the London wavelength 10^3\AA . The energy can thus be calculated from the expression for the attractive energy between two equal spheres of radius a at a minimum separation H , (Kruyt, 1952)

$$V_{att} = - \frac{A a}{12H}$$

The/

The values for the energy are given in Table VIII. They cover the range $0.75 - 1.8 \times 10^{-11}$ ergs, which can be alternatively expressed in terms of kT and they are of the order of $200kT$. This is greatly in excess of the energy required for stable adhesions to occur. If these values for the London constant are used to approximate a value for the energy of adhesion in a secondary minimum with a separation of about 100\AA , value of the order of $20kT$ is obtained which, although not a strong adhesion, confirms that particles of this size can adhere in a secondary minimum situation, (of Schenkel and Kitchener 1960).

The aggregations have been described qualitatively, a full quantitative account of them would require a full description of the surface charge properties so as to take into account the repulsive energy terms. However, quantitative assessments of the energy were made at concentrations where this term should be sufficiently small so that it can be safely ignored. This condition is true probably only in $10^{-1}M$ $CaCl_2$ for the case of phosphatidyl serine, and the stable aggregates formed are possibly a result of adsorption of ions in a stern layer. Other factors which have been ignored in this account of the aggregation/

aggregation and adhesion of phospholipid spherules are the effect of the ionic head groups in structuring the boundary layer of water in the form of "soft ice" (Derjaguin, 1964) and possible steric effects (Napper 1967) and Born repulsions (Kruyt 1952).

The former possibility has been examined by Johnson et al (1966) who concluded that deviations from the DLVO theory in the flocculation of polyvinyl acetate sols could be explained by structuring of water next to the particles providing an additional repulsive potential. Steric factors resulting from large head groups at the surface of particles give an additional entropic repulsion term. It is very difficult to make allowances for either of these terms, and again it is probable that their contribution to this system is not great as no significant deviations were observed which would require their invocation.

The final question to be asked is whether these results can be applied to cells. The behaviour of cells has been primarily studied by means of model systems utilising tissue culture methods (Weiss, P.1947 Abercrombie and Heaysman 1953, 1954, Steinberg 1963, Roth and Weston 1967). Model systems have contributed considerably to our knowledge of cell behaviour and from the very nature of the living organism/

organism are a necessity rather than a convenience. However, when we consider the results of model systems, we are one step removed from the living organism and therefore the results must be treated with circumspection. In this study, caution is very definitely "the password of the day" as it moves one step further and uses a model of the cell itself.

However, as was seen in the "Introduction", phospholipid spherules are thought to be formed of structures which are similar to the membranes of cells. Although these obviously "fall far short of the real thing" (Bangham 1968), the hope is that parameters measured on such a system can be applied to the cell membrane. As the primary site of cell contact reactions is the membrane, it is further hoped that the parameters may be useful in an analysis of cell contact behaviour, in particular adhesion.

The measurements of the London-Hamaker constant, A , are of interest with respect to cells. As this London force is approximately additive, the terms for the phospholipid constitute only one contribution to the total term. But they probably constitute the major contribution as the bulk of the periphery of the/
the/

the cell is phospholipid and the London-Hamaker constants might be expected to be comparable. The contribution of other components is anyway very difficult to quantitate (but see Vold, 1961). Measurements have been made of the London-Hamaker constant for various cell types. Wilkins, Ottewill and Bangham (1962b) using Fuchs' treatment derived a value for A for sheep leucocytes of 1.6×10^{-14} ergs. Curtis (1969) using the technique detailed here derived values for a number of cell types in the range 10^{-14} - 10^{-15} ergs. The values measured for both phospholipid are thus very similar, not only to one another, but also to the value measured for cells. It is thus possible that the phospholipid is of primary importance in the adhesion of cells. The fact that both phospholipids have similar values, would indicate that any alteration in the type of phospholipid constituting the membrane will have little effect on the attractive interaction, although significant proportions of charged phospholipids may result in considerable surface potentials and hence an increase in the energy of electrostatic repulsion (Bangham, 1968), quite apart from the action of other charged groups on the surface of the membrane.

The/

The calculations of the energy of adhesion are also possibly applicable to cells as the spherules are of a similar size range to cells in suspension. The values for the adhesion in the primary minimum, in excess of 100KT, indicate that these are very stable adhesions and are comparable to those cases where cells are thought to adhere in the primary minimum, for example sheep leucocytes (Wilkins, Ottewill and Bangham 1962), chick neural retina cells and various tissue culture lines (Curtis 1969). Such a value for the London constant would also allow adhesion of cells in a secondary minimum (Curtis, 1966, Brooks et al, 1967) with an energy of the order of 20KT on the theory of cell adhesion due to Curtis (1966). Thus the phospholipids of the plasmalemma may be a key parameter in determining the attractive force between cells.

The action of the different ions, although the evidence is somewhat tenuous, is also of interest. The greater efficacy of calcium ions in promoting aggregation and their presumed binding by phospholipids is of particular interest. Calcium ions have long been considered of key importance in cell adhesion (Steinberg 1958, Bangham and Pethica 1960, Curtis 1962) although/

although the claims of potassium have been advanced (Rappaport and Howze 1966). A number of studies have been made on the ability of other divalent cations to substitute for calcium in adhesion. The results obtained vary with the cell type although calcium is the most generally effective ion (see Table in Armstrong and Jones 1968), the most significant exception being the sheep polymorphonuclear leucocyte which is most responsive to Barium (Wilkins et al 1962a). It has been generally assumed that the calcium binds to carboxyl groups on the cell surface, although the carboxyl groups which have been measured do not bind calcium very strongly ($pK \approx 1.5$, Sillen and Martell 1964) and it has been proposed that binding also involves co-ordination with substituted amino groups as in chelating agents (Armstrong and Jones 1968). However, there is no evidence for such a mechanism. These results and those of Bangham and Papahadjopoulos (1966) on the binding of calcium to phosphatidyl serine, which ion it binds more strongly than magnesium, may indicate that the binding by phospholipids of calcium may be of greater importance in a study of cell adhesion than has been previously thought.

Less/

Less speculatively the binding of calcium by phospholipids has relevance to its effect on the action potential of nerve cells (Katz 1966), on the permeability of model membranes (Bangham 1968) and on the junctional uncoupling of cell membranes (Loewenstein 1966).

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