Contact Interactions in Phospholipid Model Membrane Systems

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

ABSTRACT

Considerations of the physical forces of interaction between phospholipid lamellae are relevant to the problem of contact between cell membranes. The lamellar phase of lecithin in water, consisting of bimolecular layers of lipid alternating with aqueous layers, is a useful model system for studying such interactions. Balance between repulsive forces and long range Van der Waals forces limits the amount of water that can be taken into the structure.

The repulsive forces between bimolecular leaflets of lecithin has been changed by the addition of a charged lipid. With 11 mole per cent of CTAB (stearyl trimethylammonium bromide) in the lecithin, the aqueous layer thickness, d_w, increased by 33Å. The dielectric properties of the aqueous space has been changed by the use of glucose, sucrose, and dextran solutions. With the addition of sucrose or glucose, the lamellar repeat distance, d, went through a maximum at 22 weight per cent sugar solution, reflecting a minimum in the attractive forces between bilayers at this concentration. With the addition of dextran, the lamellar repeat was observed to decrease by 7Å over the range of solutions used. The structure of the

lipid bilayer did not change in the above experiments save with dextran. Here, a 4 weight per cent solution appeared to be effective in decreasing the bilayer thickness d_1 by about 8 Å. The change in attractive forces with sucrose and glucose solutions are in qualitative agreement with the calculations of Van der Waals forces in lipid-water systems developed by V. A. Parsegian and his colleagues.

In an effort to find an experimental model system for the study of contact interactions in a lipid-protein system, precipitates of a synthetic protein poly-l-lysine with varying mixtures of lecithin and phosphatidyl inositol, were prepared. A lamellar structure was formed consisting of alternating layers of lipid and protein plus water, in which the interactions between the protein and lipid appeared to be strictly ionic. The amount of protein bound and the charge density in the precipitates remained relatively constant as the lecithin content was increased, while the water content, measured as an aqueous layer thickness, increased by 20 Å over the same range. The system proved unsatisfactory for the study of contact interactions as the protein appeared to be binding to adjacent bilayers thus preventing swelling.

GENERAL INTRODUCTION

One of the major problems in biological research is that of the structure of cell membranes and the nature of their interactions. Because of their extreme thinness and liability, biological membranes are difficult to study. The main molecular components of membranes are lipids and proteins. There has been much debate over how these combine to form a membrane structure.

Overton (1895) first demonstrated the lipoidal nature of the cell membrane. He found that the permeability of non-electrolytes was proportional to their oil-water partition coefficient. Bragg (1924) and Langmuir (1917) were able to show that lipoidal material has a tendency to form monolayers and bilayers. Gorter and Grendell (1925) extracted lipids from erythrocytes and using a Langmuir trough equipped with a copper strip to bring the molecules into contact, measured the surface area of a monolayer of the extracted lipids. In order to calculate the surface area of the erythrocytes they measured their number in a counting chamber and their average cellular area with a microscope. They discovered that the surface area of the extracted lipid was sufficient to cover the erythrocytes twice. This suggested that the lipid was arranged in the form of a bimolecular layer. On the basis of surface tension measurements,

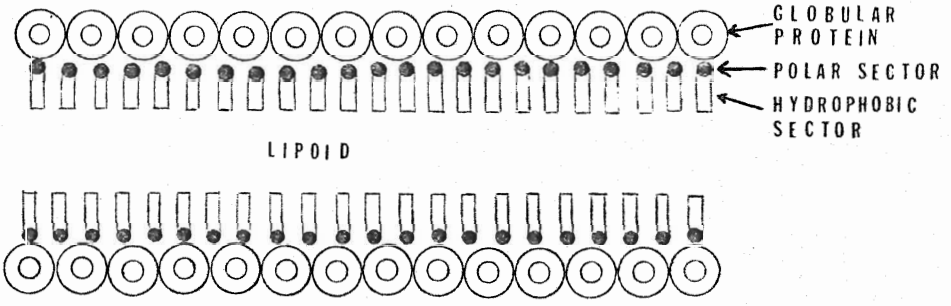
Danielli and Davson (1935) originally proposed that proteins must be absorbed at the lipid-water interface. They advanced a"pauci-molecular" model for the membrane that consisted of a lipid bimolecular leaflet coated on either side by units of globular protein (Fig. I). Later, Robertson (1957, 1958, 1960) proposed another bilayer model based mainly on evidence from optical polarization, electron microscopic and x-ray diffraction studies on nerve myelin sheath. In his model, the membrane was asymmetric. On the extracellular face of the lipid bilayer there was thought to be mucopoly-saccharide. The intracellular face was thought to be coated with protein in the extended β conformation (Fig. II). Since electron micrographs of fixed and stained cell membranes showed a triple layered structure, Robertson felt that his model applied to all membranes. He called it the "unit membrane" hypothesis.

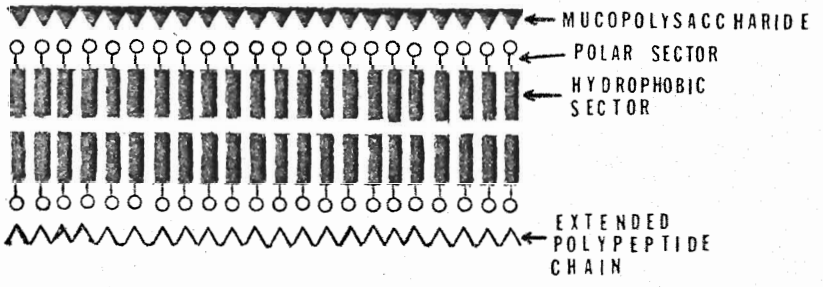
After a time, the validity of the bilayer model was challenged by several authors (Sjostrand 1963, Korn 1966, Green and Perdue 1966, Parsons 1967). It has been shown that the unit membrane structure of fixed mito-chondrial and myelin membranes, displayed in electron micrographs, remained intact even after the lipid was extracted (Fleischer, Fleischer, and Stoeckenius 1967, Napolitano, Lebaron and Scaletti 1967). Green and Fleischer (1963) demonstrated that structural protein extracted from mitochondrial membranes formed complexes

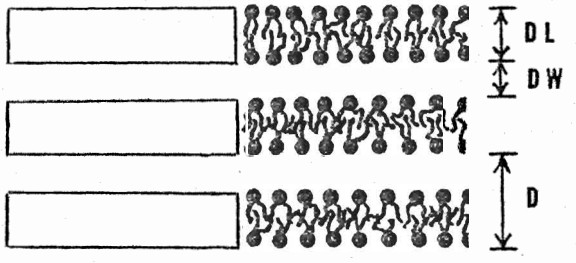
Fig. I. Davson-Danielli model of biological membrane. (Original copy: Danielli and Davson, J. Cell. Comp. Physiol. 5: 495, 1935)

Fig. II. Diagrammatic representation of unit membrane model of Robertson. (Original copy: Robertson, in 'Cellular Membranes in Development', M. Loch, ed., Academic Press, 1964)

Fig. III. Diagrammatic representation of the lamellar phase in lipid water systems.


GLOBULAR PROTEIN
POLAR SECTOR
HYDROPHOBIC SECTOR
LIPOID


MUCOPOLYSACCHARIDE
POLAR SECTOR
HYDROPHOBIC SECTOR
EXTENDED POLYPEPTIDE CHAIN


DL
DW
D

with lecithin, a neutral phospholipid. Complexes between this structural protein and synthetic alkyl phosphates could not be dissociated by salt solution, while cytochrome C, after binding to the above complex could be dissociated by salt. This suggested that the structural protein binds hydrophobically to the alkyl phosphate leavingthe ionic groups free to bind cytochrome C. Electron microscopy of mouse kidney cytomembranes and mouse kidney mitochondrial membranes and x-ray diffraction and electron microscopy studies of frog retina membranes reveal the presence of globular repeating structures, (Sjostrand 1963, Blasie and Dewey 1965). Spectroscopy indicated that much of the protein in membranes was in the α helical form rather than the pleated sheet (Wallach and Zahler, 1966, Lenard and Singer, 1968). On the basis of this evidence various subunit models for the membrane structure were advanced.

In the subunit model, the proteins are a more integral part of the membrane structure. Since some membrane proteins are enzymatically active, this model has functional advantages. From an energetic standpoint the main difference between the bilayer and subunit models is that in the former, the interactions between lipid and protein are primarily ionic and polar, while in the latter they are largely hydrophobic.

Singer and Nicholson (1972) proposed a bilayer

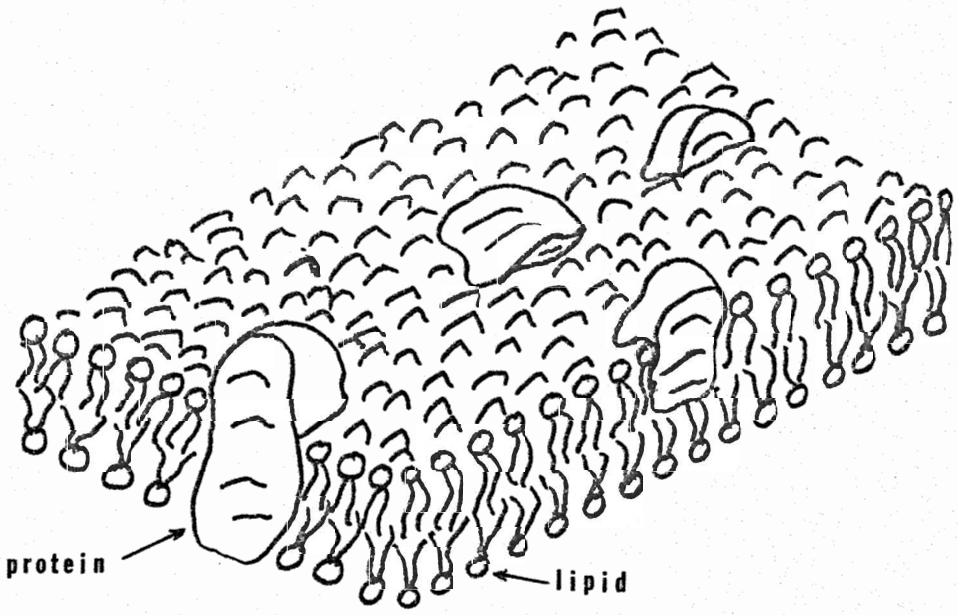
model where there could be extensive hydrophobic bonding between lipid and protein. They believed that some protein, held by ionic bonds would lie on the surface, while other more hydrophobic proteins would penetrate into and through the bilayer. The hydrocarbon tails of the lipid were thought to be melted so that the structure would be very fluid (Fig. IV).

It should be kept in mind that although there are many structures proposed for the membrane they are not necessarily mutually exclusive. It is quite feasible that different areas of the membrane could have different structures and that these structures could change in response to local physico-chemical conditions.

The topic of the structure of the cell membrane has been reviewed rather completely elsewhere, (Kavanau 1965, Stoeckenuis and Engleman 1969, Korn 1966, 1968, Hendler 1971).

In addition to membrane structure, membrane contact phenomena have been the object of much study. A fundamental and important property of all cells that is an important factor in many interactions is the ability to adhere. For example, during embryogenesis specific layers of cells adhere and differentiate to form tissues and organs. Differential contact between these cells then is a determining factor both in a cell's fate and in the development of structural organization. Certain cells, such as red blood cells and amoeboid cells, will not adhere to one another. Most cells can be made

Fig. IV. Singer-Nicholson fluid mosaic model of the biological membrane. (Original copy: Singer and Nicholson, Science 175: 720, 1072)


protein
lipid

to adhere to some surface such as plastic or glass. The specificity of contact between cells is thought to be dependent on the composition of the membrane and the nature of the bathing media (D. Gingell, 1973). Much of the material of this thesis has been devoted to the study of contact phenomena by means of changing both the membrane composition and the bathing media of certain model membrane systems.

The study of cell membranes by the use of model systems provides certain advantages over the study of intact heterogeneous membranes. The systems can be made chemically and structurally homogeneous. Many variables can be controlled so that the resulting interactions can be assessed in relation to specific changes. Information gained on the nature of these interactions can be used to offer explanations for the behaviour of real membranes.

The liquid crystals formed when lipid is mixed with water are some of the model systems often used for membrane studies. These liquid crystals have been known for a long time, to the investigators in the soap and detergent field. Much of the work in this area was done by McBain and his school (1924, 1925). The first analysis by x-ray diffraction of soap mesophases was done by Stauff (1939) and by Kiessig and Philippoff (1939). Liquid crystals obtained with phospholipids in the presence of water were examined

by x-ray analysis in 1941 by Schmitt et al. More recently, Luzzati et al (1962, 1968) defined the structure of different mesophases with more precision. They described the phases formed by various lipids in water and expanded these studies to lipid-protein systems. It was formerly thought that in the lipid-protein systems, only lamellar structures of the Davson-Danielli type would be encountered. Luzzati, however, discovered that many new phases appeared in the presence of proteins. This is a strong indication that such polymorphism could occur in real membrane structures.

Other methods used to study liquid crystals are nuclear magnetic resonance, electron spin resonance, flourescent probes, and electron microscopy. In this study, the technique of x-ray diffraction has been used exclusively. In the first part, a lecithin water system has been used as a model system for studying the forces involved in cell contact behaviour. In the second part, a poly-l-lysine, lecithin, phosphatidyl inositol system was used to study lipid-protein interactions.

Part 1

INTRODUCTION

There are two general viewpoints regarding the nature of cellular adhesion. In one, it is thought that cells adhere through intercellular bonding by "cementing" substances. Proteins or glycoproteins on adjacent surfaces are thought to bond together in a "lock and key", (or enzyme-substrate), fashion. In the other, cells are viewed as rather large colloid particles where long range physical forces determine the contact interactions (Gingell, 1973). In the latter interpretation, it is not necessary for the cell surfaces to be in molecular contact in order to interact. A general theory on the stability of lyophobic colloids has been developed by Verwey and Overbeek (1948). This theory can be used as a basis for rationalizing the interactions occuring in lipid liquid crystal systems.

In the structures formed by amphiphilic substances such as lipids, the molecules arrange themselves such that the hydrophilic head groups form an interface with the water. One of the most common phases formed in such systems is the lamellar phase. Here, planar, equidistant layers of amphiphilic molecules are separated by an aqueous gap. The hydrophilic portions of the molecules

are oriented towards the aqueous layer while the hydrophobic portions, (usually hydrocarbon), are buried within the interior. If the amphiphilic molecule has a single terminal hydrophilic portion, the layer will be two molecules in thickness. Such a layer is termed a bimolecular leaflet. (See Fig. III). This is the type of structure formed by the lipids investigated in this study.

In the case of ionizable lipids, the charges on the head groups will cause an electrostatic potential to be set up at the interface. Counterions are attracted to the interface and an electrical double layer is established. In the first few angstroms of this layer, the counter ions are absorbed into a compact region called the Stern layer. In the next few hundred angstroms, the counterions are loosely held and free to move. This is called the Gouy-Chapman layer. A shear boundary exists between these regions (Fig. VI). The electrostatic potential at the shear boundary is called the Zeta potential. It falls off exponentially away from the interface and varies as the inverse of the dielectric constant of the medium.

In addition to this repulsive force between layers of head groups across the water space, there exists a Van der Waals attractive force between adjacent layers. In the case of lamellar structures the Van der Waals potential varies roughly as the square of the inverse of the distance between layers. Since the Zeta potential falls off expo-

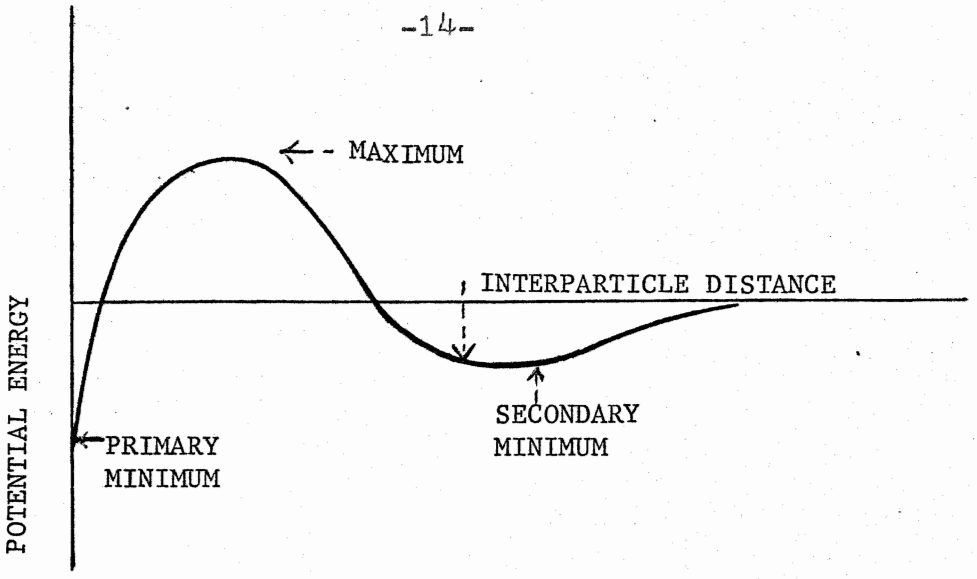
nentially, the attractive forces, provided they are large enough, will become larger than the repulsive for large distances. The sum of the attractive and repulsive potential gives an energy curve with three regions (Fig. V). The primary minimum is caused by the strong Van der Waals contact potential that exists between adjacent atoms. It varies as the inverse of the sixth power between atoms. The secondary minimum is caused by the longer range Van der Waals attractive potential. When the secondary potential minimum is of greater magnitude than thermal energy, (KT), dispersion formations will be stable.

Such systems can also be treated thermodynamically. The uptake of water into a liquid crystal can be considered in terms of an osmotic force that is related to the chemical activity of the head groups (Luzzati, 1968). Parsegian, (1967) used a thermodynamic surface coefficient, δ , to analyze the uptake of water by egg lecithin. Such a treatment is perhaps useful for lipids that contain no net charge such as monoglycerides. Theoretically, osmotic forces should be taken into account by a complete treatment of all the electrodynamic interactions that arise within the media (Parsegian, 1970).

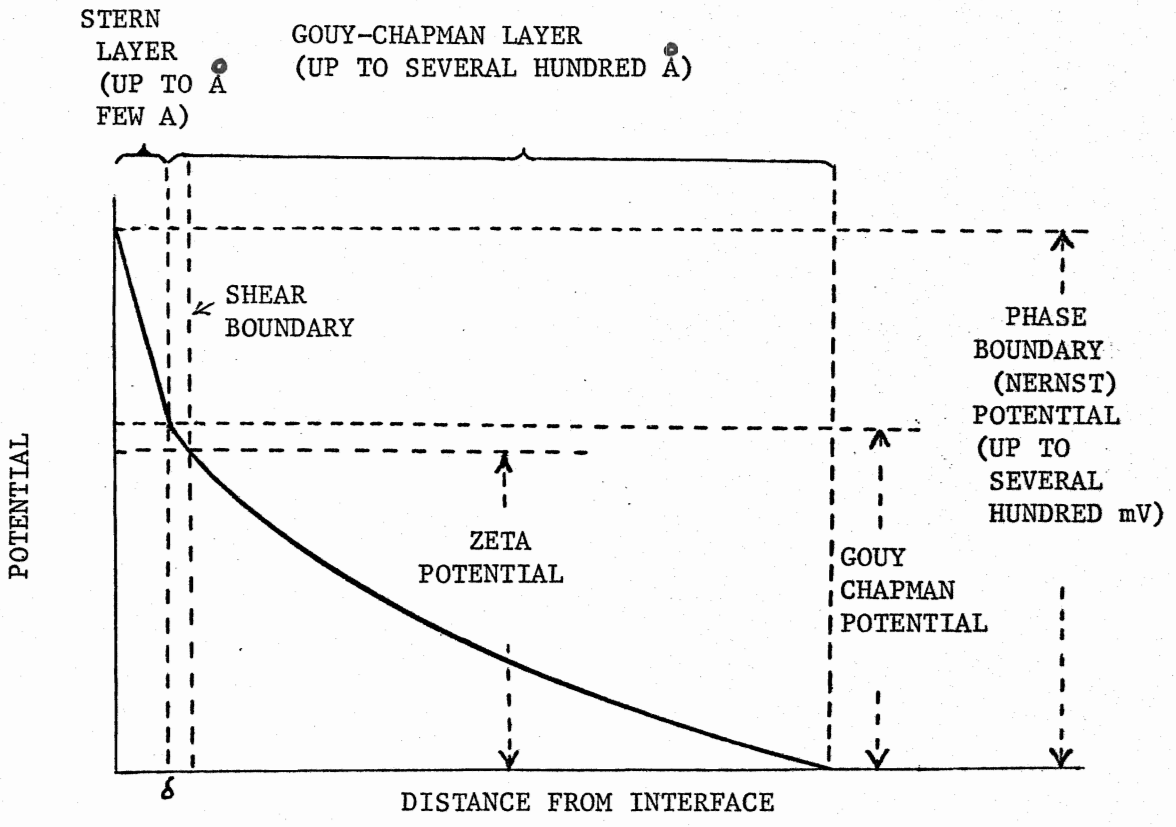
It is thought that such analyses can be used to explain cell-cell interactions. Weiss (1967) stated "All cells from vertebrates so far examined carry a net negative surface charge. Contact interactions between such cells may be usefully considered in terms of balance

Fig. V. Potential energy curve of the type that would describe the interaction of two similarily charged membranes across a planar gap. (redrawn from Kavanau, in 'Structure and Function in Biological Membrane', Holden-Day, San Francisco, 1965)

Fig. VI. Diagrammatic representation of the potential energy as a function of the distance from the interface for a planar electrical double-layer (redrawn from Kavanau, in, 'Structure and Function in Biological Membranes', Holden-Day, San Francisco, 1965)



Curve illustrating the magnitude of the total potential energy of interaction between two similarly-charged colloidal particles as a function of the interparticle distance.



A schematic graphic representation of double-layer relationships according to the theory of Stern (1924). The fixed charges and counterions are not shown. The phase boundary or Nernst potential also is known as the total interfacial potential. The shear boundary (a slipping layer of finite thickness) is shown arbitrarily in the Gouy-Chapman layer but could be at δ or even inthe Stern layer.

of electrostatic repulsion and attractive interactions of the London Van der Waals type." He explained the strong and weak adhesions to glass exhibited by two different cell cultures in terms of the primary and secondary minima in the potential energy curve. The 100-200 angstrom spaces, often shown between membranes of different cells in electron micrographs (Johnson, 1970), supposedly depend on the position and depth of the secondary minimum.

In order to gain a deeper insight into cell contact behaviour it is worthwhile to look, briefly, at the Van der Waals force in more detail. Van der Waals forces arise from transient electrical polarization due to distortions of electron clouds, molecular distortion and molecular orientation. Estimates of dispersion energies have been made in the past using the London theory (Verwey and Overbeek, 1948). It contains some assumptions such as the pairwise additivity of individual interatomic interactions that can lead to quite erroneous results for condensed systems. (Ones where the range of strong interactions exceeds the distance between atomic centres). E. M. Lifshitz (1955, 1960), developed a theory that treats the material involved as a continuum, and analyzes the fields that can arise within the system. Parsegian and Ninham, (1970), have been able to apply this theory to consider the interaction energies arising in hydrocarbon films in aqueous media,

and other systems of biological significance. This theory is briefly summarized as follows.

The electric field of a certain frequency (ω) that arises in a medium in response to an applied field of the same frequency, is characterized by the bulk dielectric susceptibility $\epsilon(\omega)$. Interaction energies that arise in systems of two or more media will depend on their dielectric susceptibilities $\epsilon(\omega)$ and on the distance between the media. The specific interaction energy that exists between two semi-infinite regions of material "1" separated by a small planar gap, of width l, filled with a second medium "2" is:

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

(This is the leading term.)

$2\pi\hbar$ is Planck's constant and $\epsilon_1(i\xi)$ and $\epsilon_2(i\xi)$ are the dielectric susceptibilities of the media evaluated on the complex frequency ($\omega = i\xi$) plane. The energy varies as the square of the inverse of the distance and is therefore long range. It is also sensitive to the difference in the dielectric susceptibilities of the media. In order to evaluate this energy it is necessary to have data on the variation of dielectric constant with frequency.

The dielectric susceptibility of polar molecules such as water changes from microwave to infared frequencies, due to Debye relaxation, and from infrared to mid

ultra violet, due to Lorentz electron dispersion. At very high frequencies, the dielectric susceptibility has a limiting value that depends on the weight density of the material. The dielectric susceptibility at optical frequencies can be found from the square of the refractive index. (n^2 and ϵ have been used interchangeably henceforth.)

During Debye relaxation the molecules become randomly disoriented from the action of Brownian movement. These disorientations can be characterized by a molecular reorientation time τ. When the frequency of the signal is of the order $\frac{1}{\tau}$ or greater, the molecules are no longer able to follow, and the dielectric constant decreases. This relaxation continues at higher frequencies, but at certain frequencies the electrons become excited and undergo damped harmonic oscillations. These oscillations can be characterized by absorption lines in the ultraviolet and infrared spectra of the molecules. (Bleaney and Bleaney, 1962)

Parsegian and Ninham (1970) were able to choose a function to describe the general behaviour of the dielectric susceptibility as a function of frequency. By combining spectral data with various simplifying assumptions they were able to use this function to calculate the Van der Waals force for certain systems.

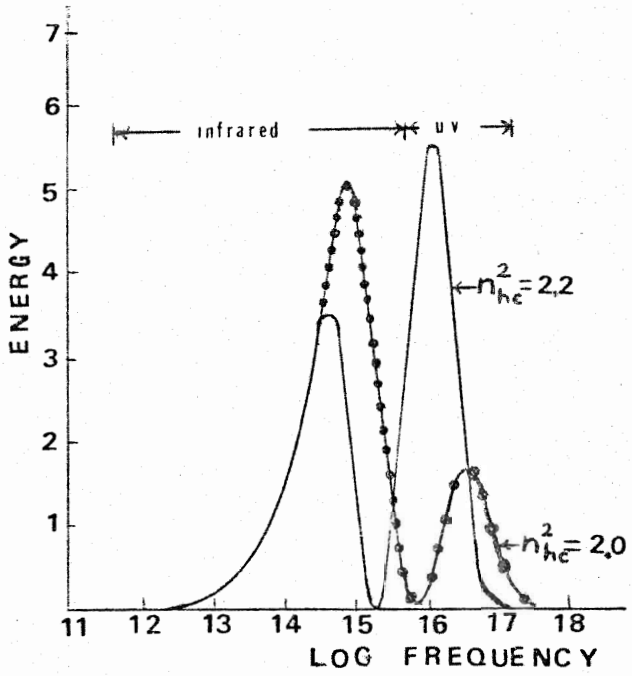
One important feature that arises from this treatment involves the fact that at high frequencies, the

dielectric susceptibility of biological molecules will all be near the same value. Since the Van der Waals force is sensitive to the difference between the dielectric susceptibility of the different media, small changes in the susceptibility at high frequencies will result in relatively large changes in the Van der Waals force. At low frequencies, the dielectric susceptibility of water is approximately eighty. At optical frequencies, it has decreased to a value of 1.78. The dielectric susceptibility of hydrocarbon, on the other hand remains essentially constant from zero frequency into the optical region, with a value of 2.0. It is only at higher frequencies when the values of ϵ_w and ϵ_{hc}, (w is the subscript for water, hc for hydrocarbon), begin to converge, that the dispersion energy becomes sensitive to these values.

Parsegian and Ninham (1969), have calculated the the interaction energy for three parallel films of hydrocarbon separated by aqueous layers. They have investigated how the energy changes as the refractive index of the water (n_w), increases. A 3% change in n_w can change the dispersion energy by 20 - 30%.

Earlier Parsegian and Ninham (1969) calculated the dispersion energy for two semi infinite media of water separated by a planar slab of hydrocarbon, for two different values of the refractive index of hydrocarbon (Fig. VII). It can be seen that it is mainly the ultra violet component of the dispersion energy that decreases

Fig. VII. Van der Waals energies for a planar slab of hydrocarbon immersed in water, for two different values of the refractive index of hydrocarbon. (redrawn from Parsegian and Ninham, Biophys. J. 10: 646, 1970)


7
6
5
4
3
2
1
ENERGY
infrared
uv
n^2_{hc}= 2,2
n^2_{hc}= 2,0
11
12
13
14
15
16
17
18
LOG FREQUENCY

as n_w^2 approaches n_{hc}^2. This illustrates the sensitivity of the energy to the high frequency dielectric susceptibility.

The Lecithin Lamellar Phase

The lecithin water system used here, for the study of force stabilization is well known. Lecithin is a major lipid component of many biological membranes (Korn, 1966). Reiss - Husson (1967), showed that lecithin in water formed a lamellar liquid crystalline phase over a wide range of temperature and concentration, (Fig. X).

The dependence of the lamellar spacing on the water concentration is of particular interest. As more water is introduced into the liquid crystal, the layers move apart and become thinner. At a certain point, the layers come to a maximal distance of separation (about 22 Å). Past this point added water is excluded from the crystal and remains as a separate phase.

Attempts have been made to explain this behaviour by considering the forces of interaction that arise between lipid leaflets. Lecithin is a neutral, zwitterionic molecule (Fig. VIII). It is debatable whether there will exist a repulsive potential between layers similar to that of the Zeta potential of ionizable lipids.

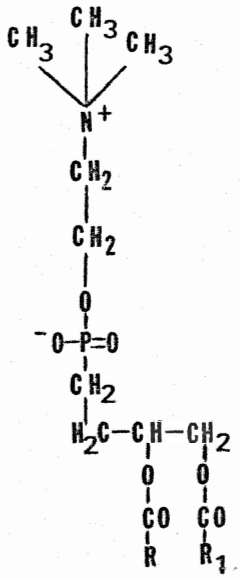
Parsegian (1967) was able to develop an internally consistent theory for the forces between bimolecular egg lecithin leaflets, by considering the $-CH_2-CH_2-N^+-(CH_3)_3$

group as a free counter ion to the phosphate group.

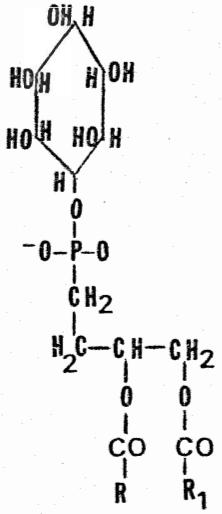
Another view that could be taken is that a repulsive potential between leaflets could be described in terms of a dipole field that arises from the two charged groups on each of the lecithin molecules. Friedenburg et al, (1966), using an idealized model consisting of a circular leaflet whose surface was studded at regular intervals with point dipoles, calculated the repulsive potential that could be expected to arise from a single egg lecithin leaflet. In order to carry out the calculations, they used an average value for the dipole moment of a lecithin molecule and for the surface area occupied by a single polar group of the molecule. They found that the repulsive energy for a sheet of infinite radius decreased as the inverse of the distance away from the sheet. (The Zeta potential on the other hand, decreases exponentially in a direction away from the surface of a sheet.) For finite radii, the energy decreased rapidly at distances larger than the radius of the sheet.

Hanai, Haydon, and Taylor, (1965), from their electrokinetic results with myelin figures of lecithin, concluded that the dipoles at the surface of the bilayer were coplanar with the surface, and that the repulsive potential was negligible. Parsegian (1967), however, carried out some calculations involving the free energy of formation that showed a repulsive potential exists between lecithin bilayers in the lamellar liquid crystal

Fig. VIII. Diagrammatic representations of the phospholipid molecules, phosphatidyl choline (lecithin) and phosphatidyl inositol (PI).



PHOSPHATIDYL CHOLINE
(LECITHIN)



PHOSPHATIDYL INOSITOL

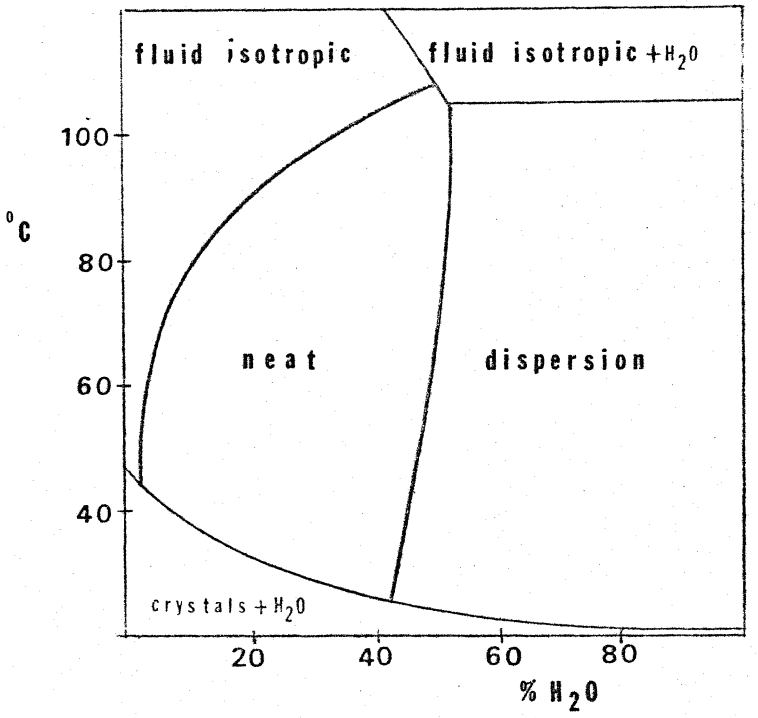
Fig. IX. Phase diagram for monocaprin in water (redrawn from Larsson, Phys. Chem. 56: 173, 1967)

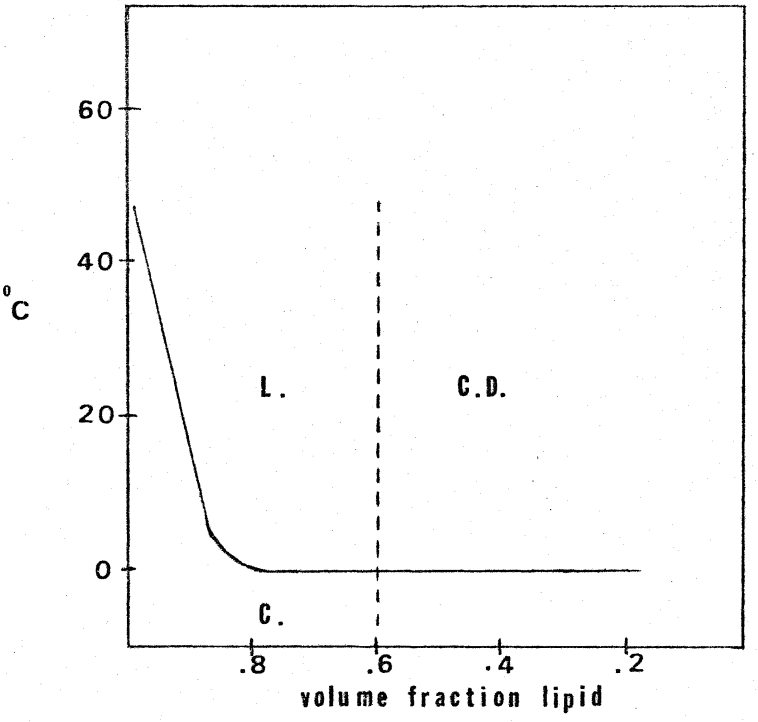
Fig. X. Phase diagram for lecithin in water (redrawn from Reiss-Husson, J. Mol. Biol. 25: 363, 1967)

L. - lamellar

C.D. - colloidal dispersion

C. - crystalline chains


fluid isotropic
fluid isotropic + H_2O
100
80
60
40
°C
neat
dispersion
crystals + H_2O
20
40
60
80
% H_2O


60
40
20
0
°C
L.
C.D.
C.
.8
.6
.4
.2
volume fraction lipid

structure, and that this potential was responsible for the thinning of the bilayer as the water concentration increased. Shah and Shalman (1965), using an ionizing air electrode measured the surface potential of a lecithin monolayer to be 240 millivolts. Although the explicit formulation remains in doubt, it is now generally accepted that a repulsive potential does exist between lecithin bimolecular leaflets.

In addition to this repulsive force, the Van der Waals attractive force is also acting in the lecithin water system, (Parsegian, 1970). The point where the layers become maximally swelled is presumeably the point at which attractive Van der Waals forces balance the repulsive. At greater distances of separation the attractive forces become stronger and the leaflets cannot move further apart. This point is described by the secondary minimum in the potential energy diagram (Fig. V). The crystal parameters, ie., the distance of separation and the thickness of the leaflets, (Fig. III), at water concentrations less than that at which the maximum separation occurs, will also be dependent on the net force between layers. This force, for d less than d maximally swelled, will be repulsive, ie., the electrostatic repulsive forces will be greater than the Van der Waals attractive.

Gulik-Krzywicki (1969) tested this theory of force balance by solubilizing small amounts of charged

detergent in the lipid of an egg lecithin liquid crystal. He found that the lipid layers moved further apart with increasing charge addition, if provided with sufficient water. At distances as great as 200 angstroms, the Van der Waals force was still strong enough to prevent further swelling. Past this point added water appears as a separate phase. This experiment provides good evidence of the long range nature of the attractive potential.

The Monocaprin Lamellar Phase

In addition to charged lipids, monoglycerides are known to form lamellar phases in water. (Larsson, 1967, Lutton, 1965). The ten carbon chain monoglyceride, monocaprin, was briefly investigated in this present study. It forms a lamellar phase over the water concentration range of 5 to 45 per cent and over the temperature range of 40 to 100°C. (Fig. IX). At high water concentrations monocaprin goes into a dispersion phase where the lamellar liquid crystals become so small as to give poor diffraction patterns. This behaviour is unlike that of lecithin in which the lamellar phase remains in equilibrium with excess water, (Larsson, 1967). Monocaprin was chosen since, of the monoglycerides, it appears to form a stable lamellar phase over the greatest range of concentration and temperature.

Plan of Part I

In part I, first the lipid layer of the lecithin liquid crystal was changed by the addition of small amounts of charged lipid. This was done in order to change the repulsive interactions between leaflets. Second, the dielectric susceptibility of the aqueous layer, (bathing medium), of egg lecithin samples was changed, by the addition of glucose, sucrose, and dextran. This was done in order to change the attractive interactions between leaflets. Sucrose solution was also added to monocaprin so that dielectric interactions could be studied for an amphiphilic molecule that carries no formal charge.

MATERIALS AND METHODS

Lecithin must be carefully handled since it will oxidize when exposed to air. It is also quite hydroscopic. Completely dry lecithin will absorb up to 30% water by weight from the air, over a period of several hours. The lipid was checked for breakdown products periodically by using thin layer chromatography. It was stored at -20°C and kept under nitrogen.

Addition of Charged Lipid to Pig-liver Lecithin

Varying amounts of stearyltrimethylammonium bromide, (CTAB), (Pfaltz and Bauer Inc.) was added to pig-liver lecithin, (Secondary Research Laboratories). 5 , 7, 9, and 11 mole per cent CTAB in lecithin mixtures were prepared by addition of CTAB in chloroform to lecithin in chloroform. The resulting mixtures were partially dried on a rotary evaporator and finally dried *in vacuo*. Water was added gravimetrically to approximately 25 milligram samples of the lecithin - CTAB mixtures to obtain the desired weight per cent lipid. These samples were allowed to come to equilibrium over a period of forty-eight hours and were then placed in x-ray sample holders. Before mounting the samples were reweighed to test that they had not dried on standing. A series of samples with 0% CTAB were also prepared as a control.

Changing the Interbilayer Space

Sucrose solutions were used to change the dielectric constant of the water layer of the lecithin liquid crystal. The pig-liver lecithin, water system used previously, proved less than satisfactory, so egg lecithin was used henceforth. The egg lecithin was extracted from fresh eggs, following the procedure of W. S. Singleton et al., (1965). Ultrapure sucrose was obtained from Swartz Bioresearch.

Sucrose solutions of varying concentrations were added gravimetrically to the lipid in sufficient amounts to cause each sample to be maximally swelled and to be in equilibrium with excess solution. The average concentration of the samples was fifty per cent egg lecithin by weight. Unfortunately, the x-ray pictures revealed that the samples prepared in this way reached equilibrium very slowly and often remained disordered. No meaningful measurements could be taken from the films so the preparation procedures were changed.

It was noticed that drier samples, in the concentration range where the single lamellar phase occurs, appeared to be well ordered. Consequently, a series of samples were prepared at approximately 70% lipid by weight. The per cent sucrose of the aqueous layer was increased from 0 to 60% by weight.

Later it was realized that because of the density differences in the sugar solutions, this last series gave

lamellar phases with different separations of the bimolecular leaflet. Hence, it would be better to mix the solutions to constant volume fraction of lipid rather than constant weight per cent. Another series, analagous to the first, was prepared at as close to .72 volume fraction of lipid as possible.

In addition to these samples, several series were prepared gravimetrically, at constant sucrose concentrations and varying egg lecithin concentration. Series from 95 to 50% egg lecithin using 0, 20, 30, 40, and 50% sucrose solutions were prepared.

In order to study another lipid of simpler head group than lecithin, monocaprin was used. A series of control samples with 0% sucrose, varying per cent monocaprin, and a series using 40% sucrose, varying per cent monocaprin, were prepared.

After some experimentation, a method was devised where the egg lecithin samples in excess sucrose solutions could be brought to equilibrium. Mixtures of egg lecithin in sucrose solutions were sonicated, for thirty seconds, at 1/3 maximal power on a Biosonik III sonicator. This procedure produced well dispersed suspensions of egg lecithin in the solutions. The samples were than centrifuged at 160 thousand g for one and one half hours. The lipid accumulated either on the top, or the bottom of tubes, depending on the density of the sucrose solutions, and could then be pipetted into x-ray sample holders.

Samples of egg lecithin in excess D^+ glucose (BDH dextrose) and dextran (BDH M.W. 200,000 to 275,000) solutions were prepared in the same way.

Drier samples of egg lecithin in dextran solution were also prepared. The dextran concentration was kept constant and the per cent egg lecithin was varied. Four, 16 and 30% dextran solutions were used in which the volume fraction of egg lecithin was varied from .9 to .4.

The density of the 4, 16 and 30% dextran solutions, used for the calculations of the lipid leaflet thickness, was determined experimentally by pycnometry.

The refractive index of the dextran was measured experimentally using an Abbe refractometer. The instrument was calibrated with liquids of known refractive index; acetone, chloroform, methanol, distilled water, and n decane.

In the above experiments, care was taken to prepare the control samples from the same lipid fraction and on the same day, as the test samples.

In order to test whether the effect of the sugar on the crystal spacing was reversible, an experiment was performed with the centrifuged samples. X-ray sample holders containg lipid in dextran, glucose and sucrose, were opened, and the contents were pipetted into centrifuge tubes filled with distilled water. The lipid was gently mixed, recentrifuged, remounted and the x-ray pictures were retaken.

X-ray Diffraction Studies

For the x-ray diffraction studies reported here, a Guinier camera operating in vacuo was used. The copper k_{α_1} line (wave length 1.540 Å) was isolated by a bent quartz crystal monochromator and was slit collimated. The samples were sealed between mica windows approximately one millimeter apart and the temperature was controlled.

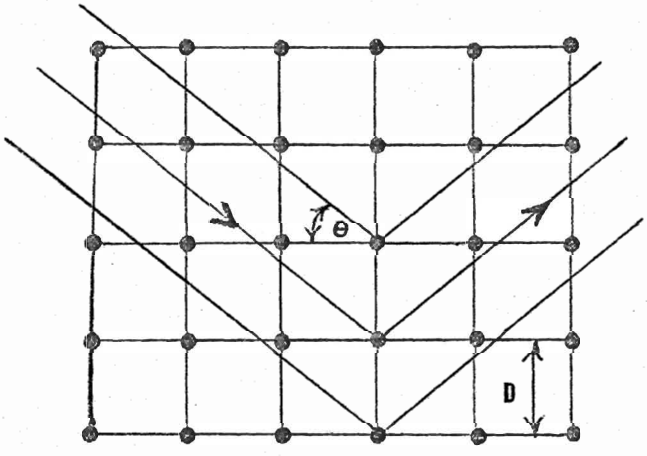
Due to their short wavelength, x-rays can be used to investigate orders on an atomic scale. Bragg interpreted scattering from crystals in terms of reflections off the various crystal planes. The scattered rays will interfere with one another forming a pattern that is related to the crystal lattice. The Bragg relation $n\lambda = 2d \sin\theta$, gives the condition for constructive interference for rays scattered at an angle θ, from a set of planes a distance d apart. (See Fig. XI). X-ray scattering from powdered crystal samples, ie., ones in which many tiny crystal fragments are randomly oriented such that all angles, θ, are represented, will result in a series of arcs or circles on the x-ray film. From the spacing and spacing ratios of these arcs, the dimensions and the type of unit cell can be determined. Since the samples used in this study are unoriented, they give a powder type x-ray pattern.

In the lamellar liquid crystals formed in the lecithin water system, there is long range order between the lipid leaflets and short range disorder among the

Fig. XI. Illustration of the Bragg scattering condition. Reflection of an x-ray beam from a series of planes passing through the lattice points of a crystal.

θ - angle of incidence of the beam on the crystal planes

d - repeat distance between planes


θ
D

molecules within the leaflet. (There is some degree of order among the hydrocarbon tails of the lipid.) These structures are essentially one dimensional crystals in that they are ordered in one direction, ie., all the leaflets are equidistant, (Fig. III). In this simple case, the dimensions of the lamellar repeat distance d can be found from Bragg's law, $n\lambda = 2d \sin\theta$, where $2\theta = \frac{l}{r}$, l is the distance between the arcs on the x-ray film and r is the distance from the sample to the film. n = 1, 2, 3 is the order of diffraction corresponding to the first, second and third lines on the film. A typical x-ray film of the lecithin liquid crystal is shown in Figure XII.

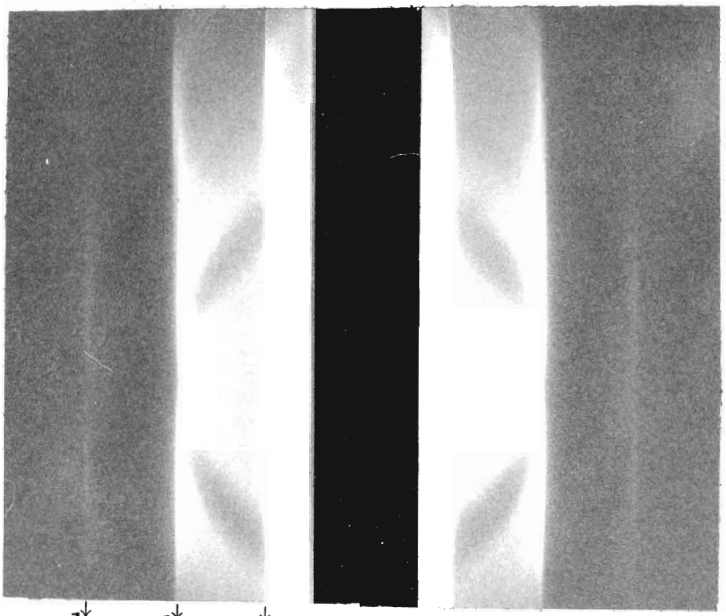
The low angle lines give the repeat distance d. A broad high angle band, (not shown), corresponding to a 4.5 Å ordering is caused by scattering from the disordered hydrocarbon tails of the lipid. The broadness of the band indicates that the chains are in thermal motion or "melted". At low temperatures the chains crystallize and true diffraction lines appear on the film, corresponding to the side packing of the chains.

X-ray Structural Determinations

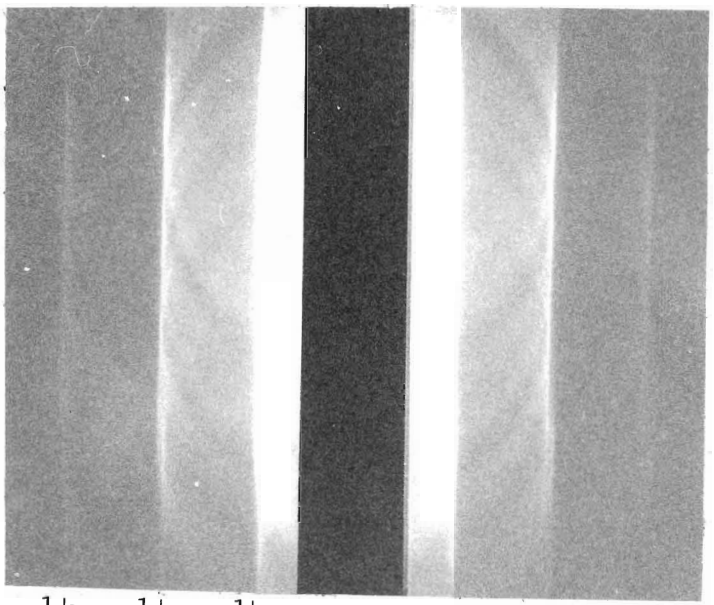
To determine the molecular packing within the unit cell of complex structures, a method involving phase determination and Fourier transformation of the scattered rays is often used. The structures formed in this study are simple enough that the molecular packing can be deduced from the known concentration and the chemical

Fig. XII. Photographs of x-ray films showing interference patterns from lipid-water samples.

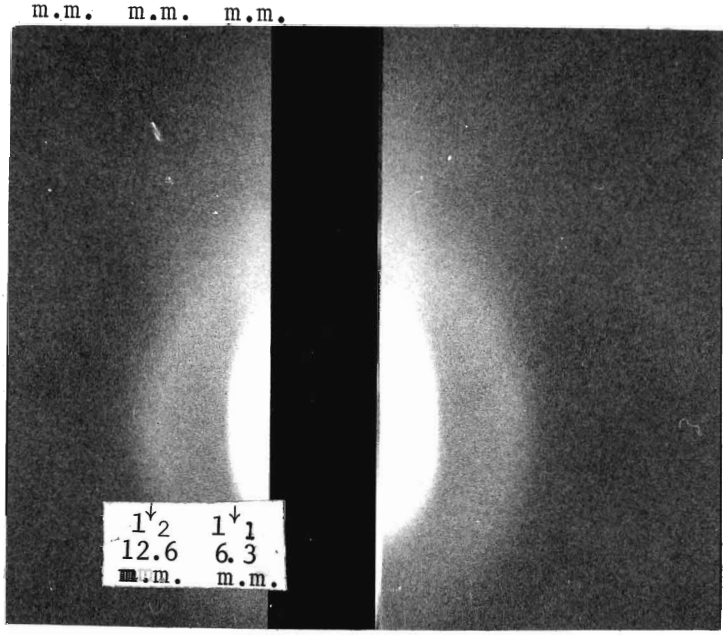
1. egg lecithin centrifuged in distilled water
2. egg lecithin centrifuged in 20% dextran solution
3. egg lecithin centrifuged in 20% sucrose solution



l_3 18.6 m.m. l_2 12.4 m.m. l_1 6.2 m.m.

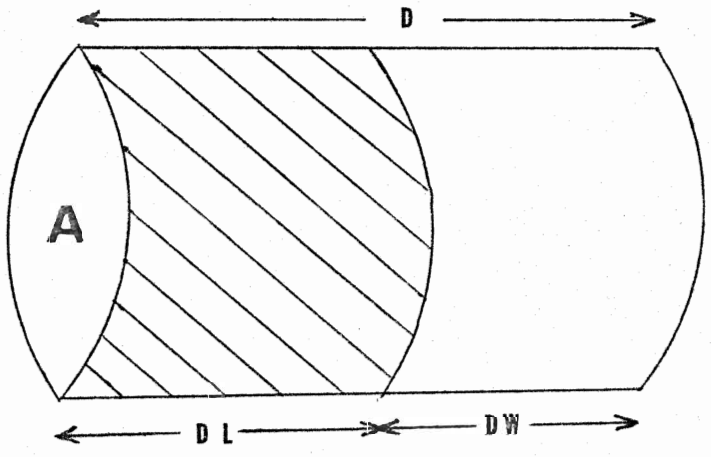


l_3 20.0 m.m. l_2 13.2 m.m. l_1 6.65 m.m.



l_2 12.6 m.m. l_1 6.3 m.m.

Fig. XIII. Hypothetical arrangement of lipid and water forming two discrete layers of thickness d_l and d_w, and surface area A.


D
A
D L
D W

nature of the component molecules, (Luzzati, 1968).

The lecithin liquid crystal consists of alternating layers of lipid and water. The thickness of the lipid layer is called d_l and the water layer, d_w. Within the lamellar repeat distance d, water and lipid must be packed in proportion to their respective volume concentrations ϕ_l and ϕ_w (Fig. XIII). These in turn are determined by the weight concentration of the components c_l and c_w ($c_l + c_w = 1$) and their partial specific volumes ν_l and ν_w.

Thus $d_l = \phi_l d$

where $\phi_l = \dfrac{c_l \nu_l}{c_l \nu_l + c_w \nu_w}$

and $d = d_l + d_w$.

The change in d, d_l, and d_w under various conditions is of interest in this study.

Note: The measurements for the data points on the graphs were taken from x-ray films of single samples prepared in a single experiment. Previous to the experiments preliminary investigations were carried out to establish the most suitable concentration ranges to be used.

RESULTS AND INTERPRETATIONS

Addition of Charged Lipid to Pig-liver Lecithin

(1) Pig-liver lecithin in distilled water

The results for the structural parameters d, d_1, and d_w of the liquid crystal formed when distilled water is added to lecithin are presented in Table I and Figures XIV and XV. The data is expressed as a function of the per cent lipid in the samples. The values for d, given in Table I, begin at a minimum of 44.7 Å at 90% lecithin and rise to a value of 69 Å at 20% lecithin. The curve labelled 0% CTAB, in Figure XIV, illustrates that there is no sharp transition to a maximally swelled value for d.

The values for d_1 (Table I) begin at 40.2 Å, at 90% lecithin and decrease to 30.3 Å at 50% lecithin. The values for d_w, (Table I), rise from 4.5 A at 90% lecithin to 30 A at 50% lecithin. No values for d_1 and d_w at concentrations less than 50% lecithin are given since it is uncertain whether or not there is excess water present in the samples. These results are illustrated graphically in Figure XIV. The results for pig-liver lecithin in distilled water serve as a control for the samples to which CTAB has been added.

(2) Pig-liver lecithin containing 5% CTAB

The values for d, d_1, and d_w plotted against per cent

Fig. XIV. Curves illustrating the dependence of d, d_1, and d^W on volume fraction of lipid for pig-liver lecithin in distilled water and pig-liver lecithin - 5% CTAB in distilled water

● pig-liver lecithin in distilled water

▲ pig-liver lecithin - 5% CTAB in distilled water

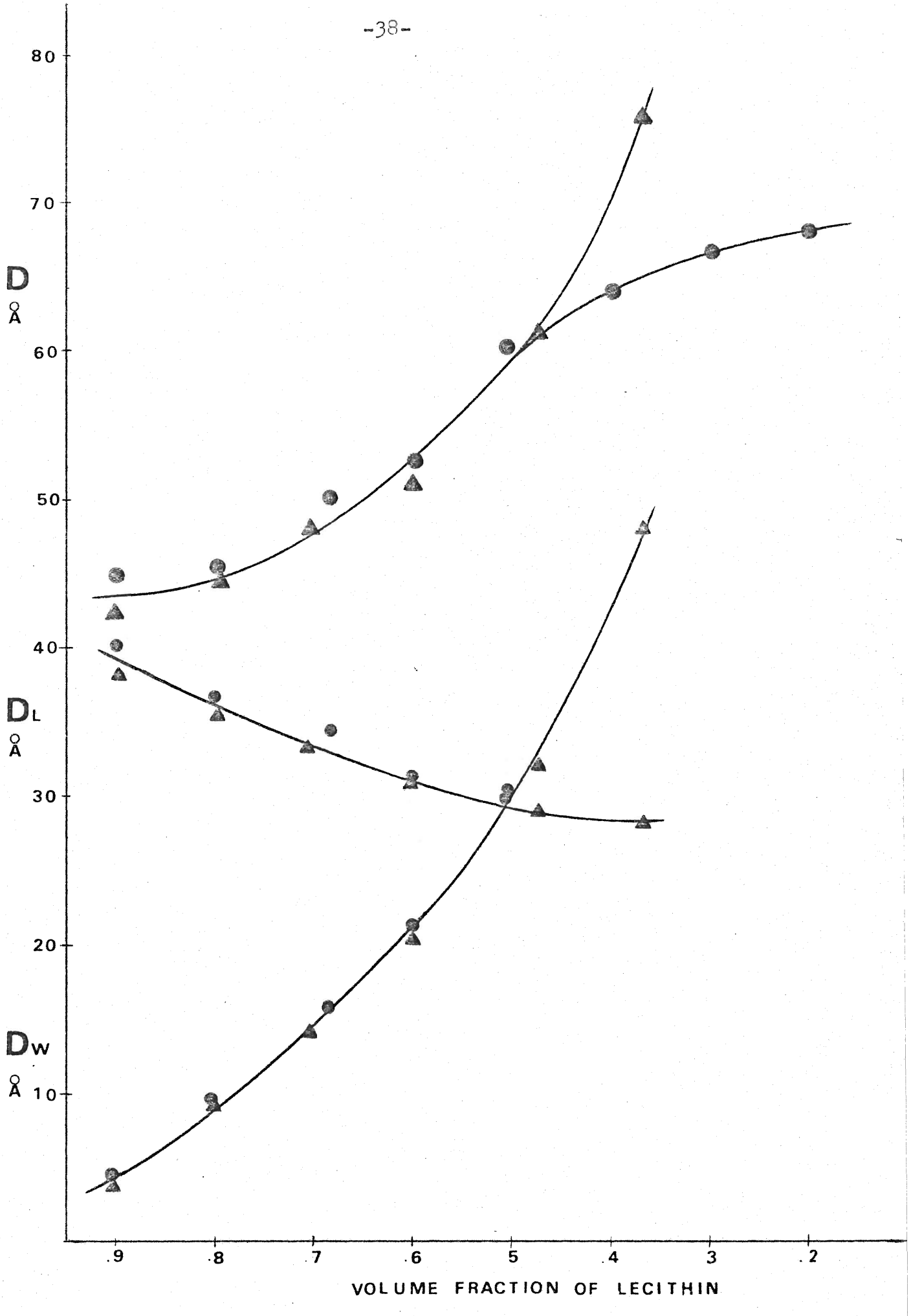

80
70
D
Å
60
50
40
DL
Å
30
20
Dw
Å
10
.9
.8
.7
.6
.5
.4
.3
.2
VOLUME FRACTION OF LECITHIN

Fig. XV. Curves illustrating the dependence of the lamellar repeat distance, d, on the volume fraction of lipid for lecithin containing varying amounts of CTAB.

● Egg lecithin in distilled water

■ Pig-liver lecithin in distilled water

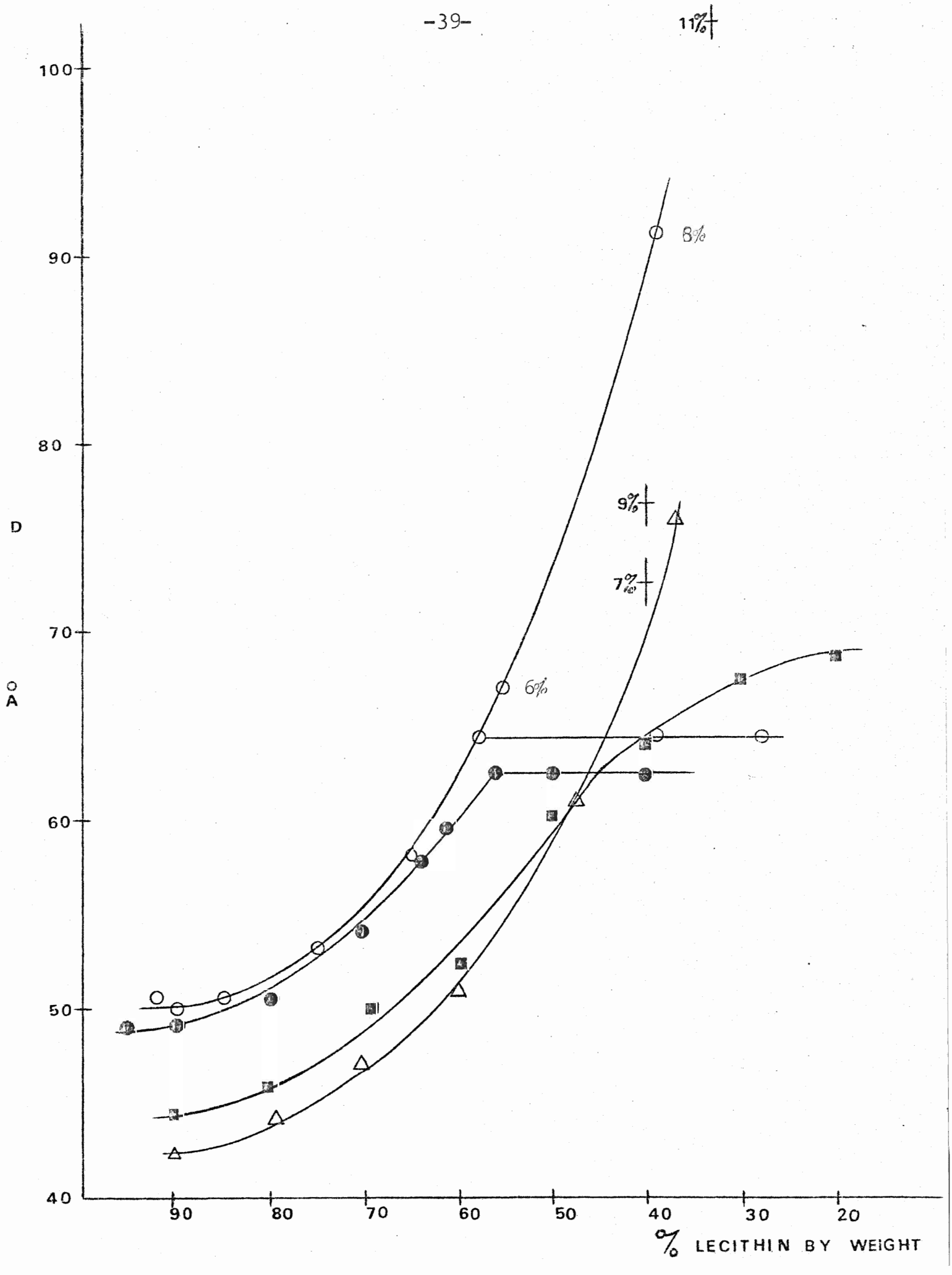
Δ Pig-liver lecithin - 5 mole per cent CTAB in distilled water

7%+ Pig-liver lecithin - 7 mole per cent CTAB in distilled water

9%+ Pig-liver lecithin - 9 mole per cent CTAB in distilled water

11%+ Pig-liver lecithin - 11 mole per cent CTAB in distilled water

○ Gulik-Kryzwicki's results for egg lecithin in distilled water and egg lecithin-6 and 8% ctab in distilled water


11%
100
90
80
D
70
O
A
60
50
40
8%
9%
7%
6%
90
80
70
60
50
40
30
20
% LECITHIN BY WEIGHT

lecithin containg 5% CTAB are given in Figures XIV and XV and Table II. The values for d, begin at 42.4 Å at 90% lecithin and rise to a value of about 76 Å at 37% lecithin. Figure XV shows that the value for d at 37% lecithin is not necessarily maximal. It represents an increase of about 7 Å over the maximal value of the control.

The values for d_1 (Table II) begin at 38.2 Å at 90% lecithin and decrease to about 28 Å at 37% lecithin. The values for d_w (Table II), rise from 4.2 Å at 90% lecithin to about 48 Å at 37% lecithin. Figure XIV shows that these values are very nearly the same as those for the control. The results indicate that the increase in the maximal value for d (76 Å), over that of the control, is due to an increase in d_w rather than d_1.

(3) Pig-liver lecithin in excess containing varying amounts of CTAB

These samples were very poorly equilibrated. The lines on the x-ray film either displayed a gradient or were very broad. Consequently, the spacings had to be estimated. The values for lecithin containing 7, 9, and 11 mole per cent CTAB are given in Table III and Figure XV. These values increased steadily as the mole per cent CTAB was increased. At 11% CTAB, d has increased by about 33 Å over that of the maximal value for the control.

(4) Egg lecithin in distilled water

Results from experiments by Gulik-Kryzwicki with

egg lecithin and CTAB are shown in Figure XV. In the control curve for egg lecithin (containing 0% CTAB), d rises from a value of 50 Å at 90% lecithin to 64 Å at 60% lecithin. At this point there is a sharp transition. For lower lecithin concentrations d remains at 64 Å and the added water appears as a separate phase. The two values of d that are above the maximal value for the control represent egg lecithin containing 6 and 8% CTAB by weight.

Control samples were also prepared from egg lecithin extracted in the laboratory. The results for these given, in Table IV, and Figures XV and XVI, are quantitatively the same as those of Gulik-Kryzwicki. The values for d rise to a maximum of 62.5 Å at 56% egg lecithin. At lower lecithin concentrations d remains constant and added water appears as a separate phase. The curve for egg lecithin shown in Figure XV should be compared to that for pig-liver lecithin shown in the same figure. The curves for pig-liver lecithin are clearly not as sharply defined as those for egg lecithin.

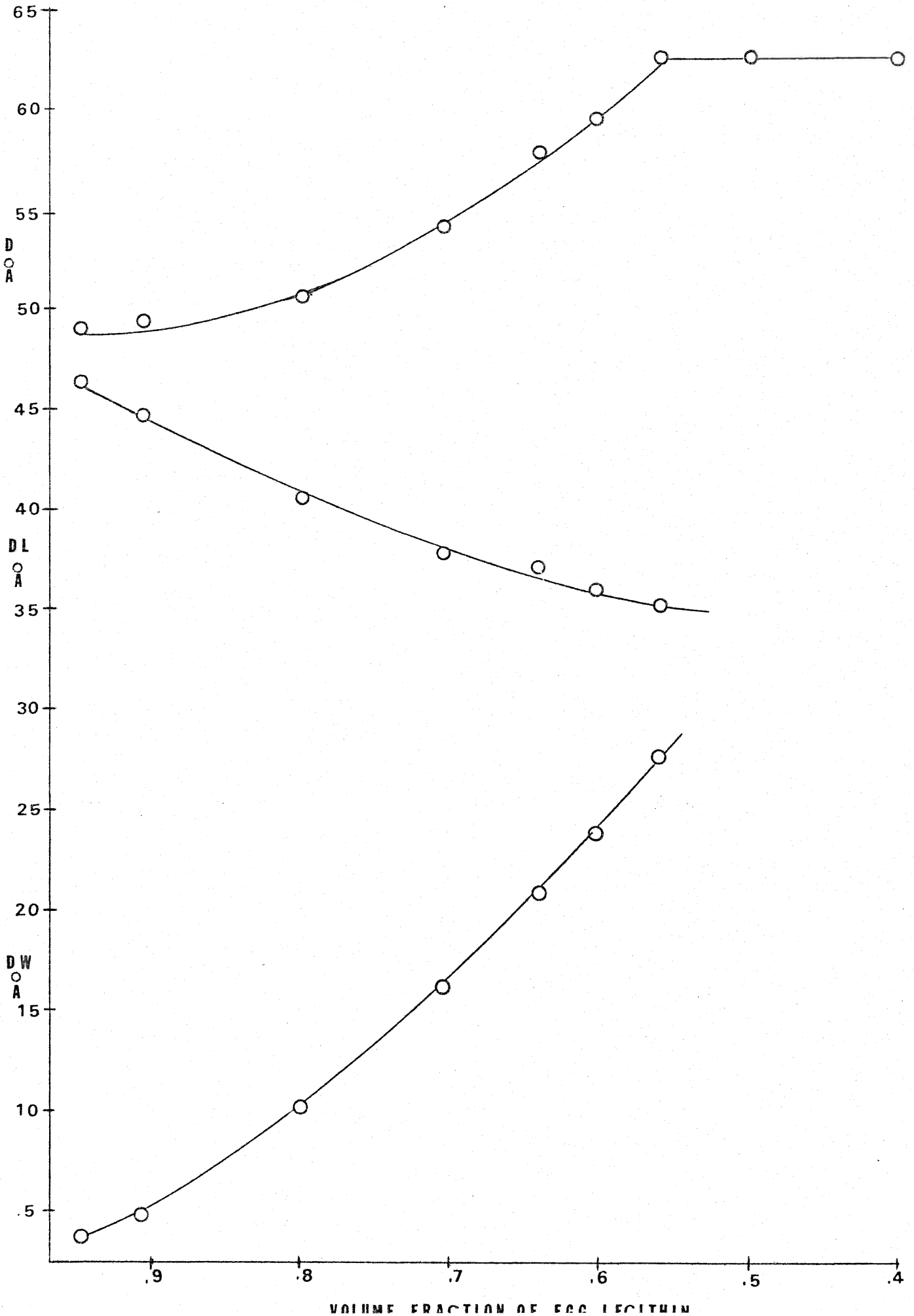
(5) Interpretation

The results obtained with pig-liver lecithin are generally the same as those of Gulik-Kryzwicki (1968) and are consistent with the idea of changing force balance between the lipid layers. As the charge on the layers increase, they move further apart if

provided with excess water. The force balance would be expected to change with added charge on the lipid leaflet in the drier samples as well as those in excess. The fact that the thicknesses, d_l and d_w, do not change could be explained by the fact that the system is constrained by the limited amount of water present.

Although the results with pig-liver lecithin were quantitatively in agreement with those with egg lecithin, some difficulties were encountered with the pig-liver lecithin system. The wetter samples were in poor equilibrium and there was no sharp transition to a constant value of d for samples in excess water. The equilibrium problems could perhaps be explained by the fact that pig-liver lecithin has a higher degree of unsaturation in the hydrocarbon tails (Gulik-Kryzwicki, private communication) than egg lecithin. This means that the tails of the pig liver lecithin molecules will be shorter than the tails of the saturated CTAB molecules even though they have on the average, the same number of carbon atoms. The longer tails of the CTAB may disrupt the molecular packing arrangements introducing disorder into the system. This effect would be greater the higher the concentration of CTAB;consistent with the observation that the lines on the x-ray pictures for the 9 and 11 per cent swollen samples were broader than those for the 3 and 5 per cent. At 3 per cent CTAB there are 32 molecules of lipid per molecule of CTAB. At 11 per cent, there are 8.

Fig. XVI. Curves illustrating the dependence of d, d_l and d_w on volume fraction of lipid for egg lecithin in distilled water.


65
60
55
D
Å
50
45
40
DL
Å
35
30
25
20
DW
Å
15
10
.5
.9
.8
.7
.6
.5
.4
VOLUME FRACTION OF EGG LECITHIN

Since the results for egg lecithin extracted in the laboratory were more satisfactory than for pig-liver lecithin, it was decided to use the egg lecithin for further studies.

Changing the Interbilayer Space

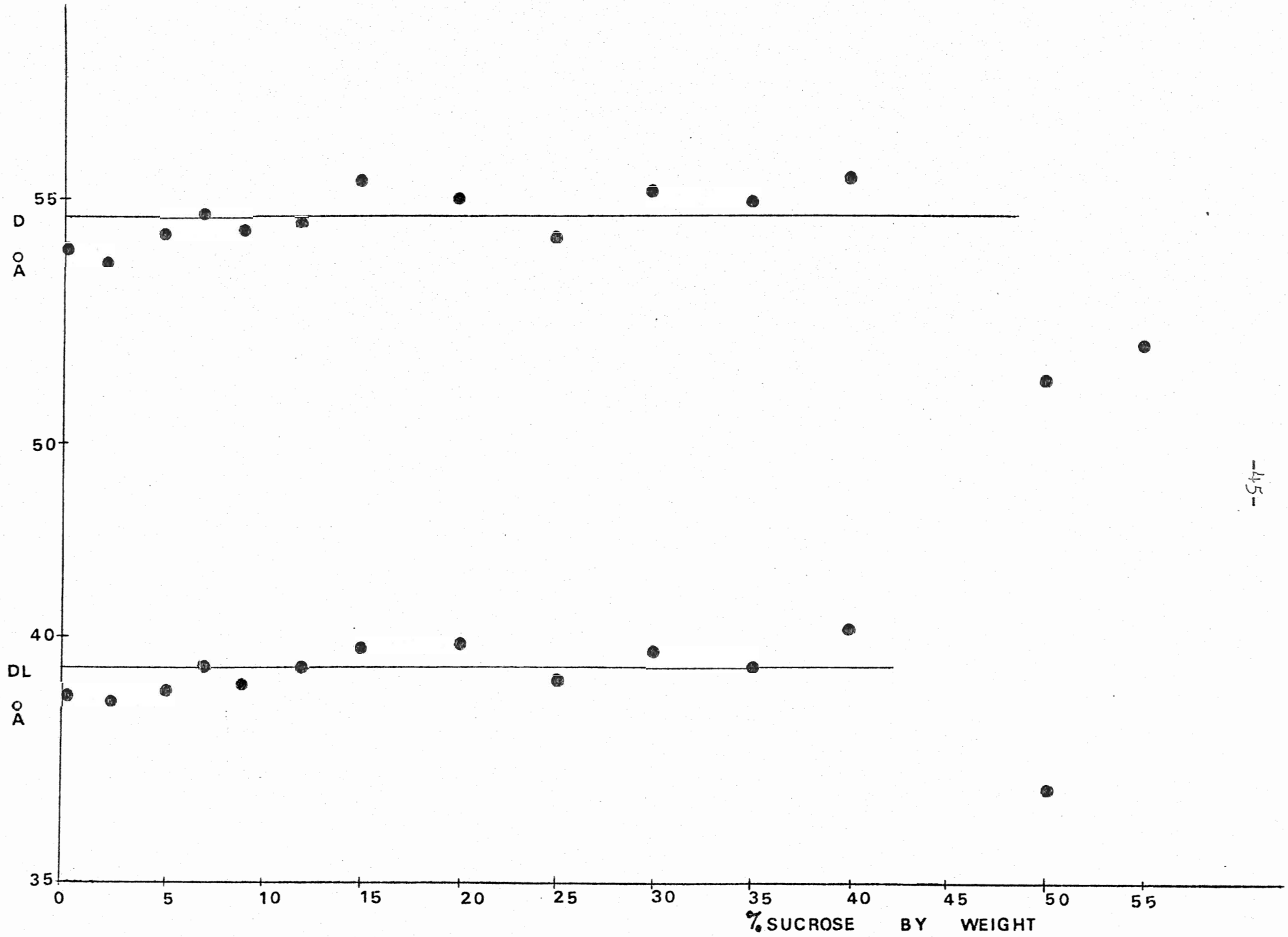
A) Experiments with Sucrose and Glucose

(1) Samples prepared gravimetrically

The data for the samples prepared gravimetrically to constant weight per cent egg lecithin (70%) are given in Table V. The per cent sucrose in the aqueous solutions used, ranges from 0 to 60%. On the x-ray film for the sample containing 60% sucrose solution, appear a series of many sharp lines indicating the presence of crystalline sucrose.

The data for the samples prepared gravimetrically to constant volume fraction (.72) egg lecithin are presented in Table VI and Figure XVII. The per cent sucrose ranges from 0 to 65%. X-ray films for the samples containing 55, 60 and 65 per cent sucrose solution indicate the presence of crystalline sucrose. The straight lines in Figure XVII indicate that, except for the sample containing 50% sucrose, neither d nor d_1 have changed with added sucrose. At 50% sucrose d and d_1 have appeared to decrease. In the samples where crystalline sucrose has appeared, no attempt has been made to calculate d_1 since the concentration of the lamellar

Fig. XVII. Curve illustrating the dependence of d and d_1 on the percentage of sucrose in the aqueous layer of the liquid crystal. The volume fraction of lipid in the samples remains constant at .72.


55
D
°A
50
40
DL
°A
35
0
5
10
15
20
25
30
35
40
45
50
55
% SUCROSE BY WEIGHT

phase is no longer known.

Data for samples in which the volume fraction of lecithin has been changed, and the per cent sucrose solution, kept constant at 30%, hasbeen presented in Table VIII and Figure XVIII. The data for the control samples containg 0% sucrose has been shown in Table VII and the same figure. Data for samples in which the volume fraction of lecithin has been changed and the per cent sucrose kept constant at 22, 40, and 50% has been given in Tables X, XI, and XII respectively. The data for the control samples for these three sucrose concentrations has been given in table IX. The results from these experiments have been combined and illustrated in Figure XIX. Figures XVIII and XIX show that until 50% sucrose solution, neither d nor d_1 have changed as the volume fraction of lecithin changes from about .90 to the point where sucrose solution is in excess. At 50% sucrose, d_1 begins to decrease. The sucrose, however, only remains in solution for the wetter samples. At volume fractions of egg lecithin higher than .75, lines corresponding to crystalline sucrose appear on the x-ray film.

(2) Samples prepared by centrifugation

Lecithin samples that are in equilibrium with excess glucose and sucrose solution have been prepared by centrifugation. Data for samples centrifuged in sucrose solutions ranging from 0 to 50% by weight are

Fig. XVIII. Curves illustrating the dependence of d and d_1 on the volume fraction of lipid for egg lecithin in distilled water and in 30% sucrose solution.

● Egg lecithin in 30% sucrose solution

o Egg lecithin in distilled water (control curve)

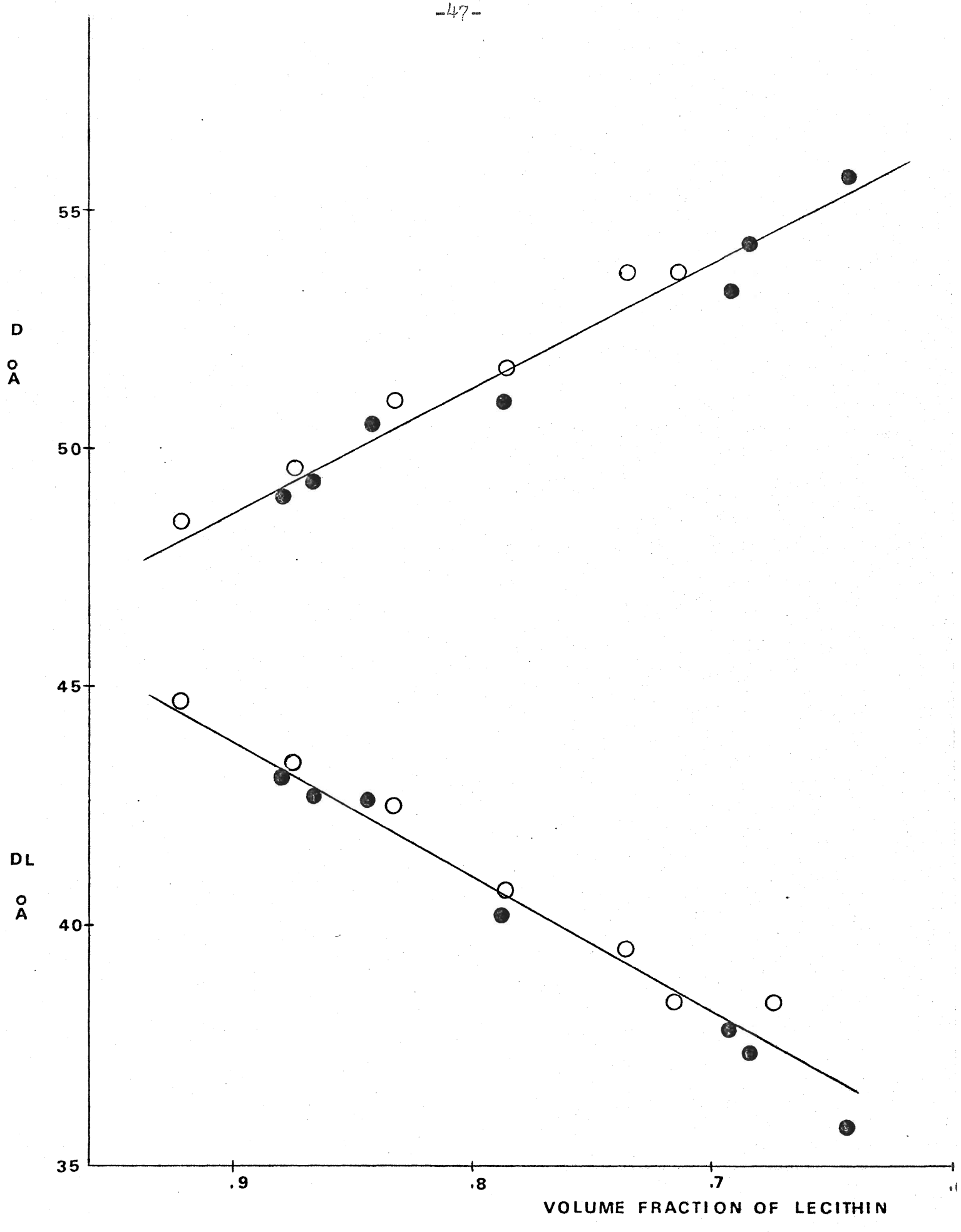

55
50
45
40
35
D
°
A
DL
°
A
.9
.8
.7
VOLUME FRACTION OF LECITHIN

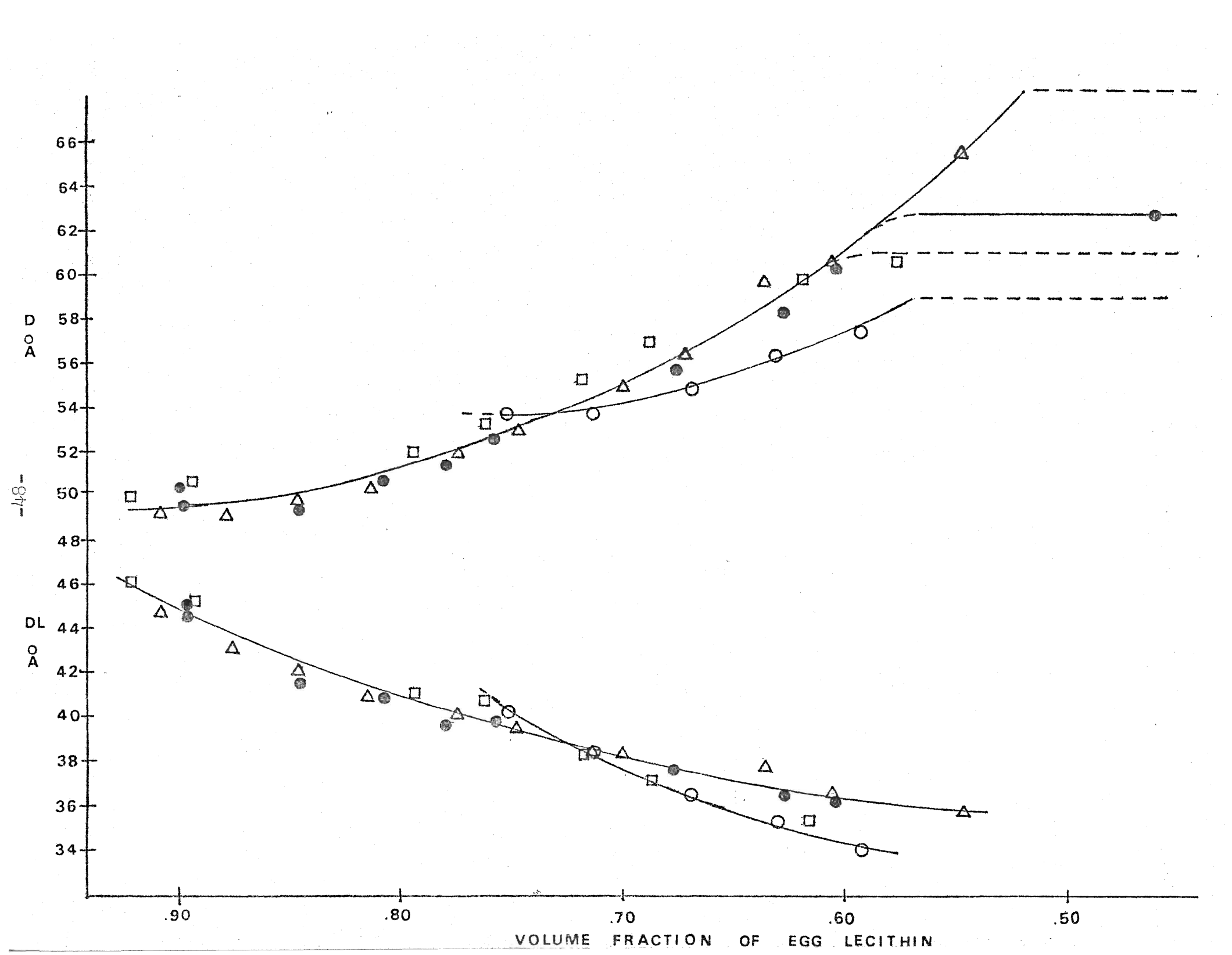
Fig. XIX. Curve illustrating the dependence of d and d_1 on volume fraction of lipid for egg lecithin in distilled water, and in 22%, 40%, and 50% sucrose solution.

● egg lecithin in distilled water (control curve)

△ egg lecithin in 22% sucrose solution

□ egg lecithin in 40% sucrose solution

○ egg lecithin in 50% sucrose solution


66
64
62
60
D
O
A
58
56
54
52
50
48
46
DL
O
A
44
42
40
38
36
34
.90
.80
.70
.60
.50
VOLUME FRACTION OF EGG LECITHIN

given in Table XVIII. Data for samples centrifuged in glucose solutions ranging from 0 to 40% are given in Table XIX. The behaviour of this data is illustrated in Figure XX. The values for d for the samples centrifuged in sucrose begin at 62.5 Å at 0% sucrose and rise to a maximum of 68.5 Å at 25% sucrose. Past this point d declines steadily. By 50% sucrose d has decreased to 58.7 Å. The values for d for the samples centrifuged in glucose behave in a similar manner. At 22% glucose d, has reached a maximum of 68 Å. By 40% glucose d has decreased to a value of 58.7 Å.

The lines on the x-ray films for the samples containing glucose and sucrose prepared by centrifugation are quite broad in comparison to the drier samples containing sucrose that have been prepared gravimetrically. The spacing between lines for the centrifuged samples, however, could be measured with nearly the same accuracy as those for the gravimetric samples, (ie. $\pm$ .5 Å).

Samples of egg lecithin that had yellowed, probably oxidized, were centrifuged in sucrose solutions. The results were compared with those from samples centrifuged with pure, freshly isolated lecithin. The data for the samples is given in Table XXI and Figure XXI. There is clearly a change in the repeat distance d as a function of the per cent sucrose as the lecithin ages.

The samples centrifuged in sucrose, glucose, and dextran that were opened and recentrifuged in distilled

Fig. XX. Curves illustrating the dependence of d on the percentage of glucose, sucrose and dextran in the aqueous layer of the egg lecithin liquid crystal. All samples have been centrifuged in the sugar solutions such that they are in equilibrium with excess solution.

● egg lecithin centrifuged in glucose solutions

△ egg lecithin centrifuged in sucrose solutions

□ egg lecithin centrifuged in dextran solutions

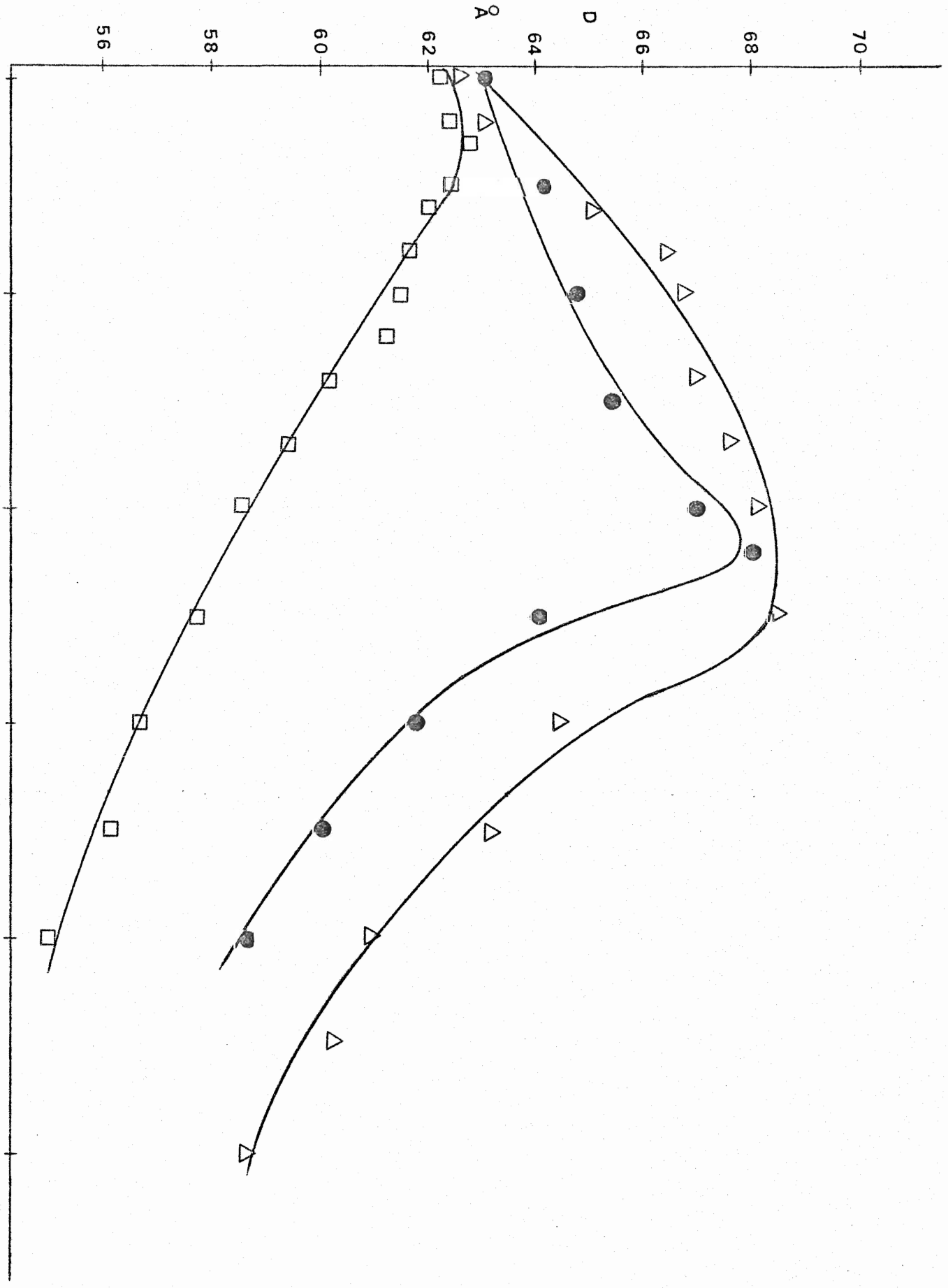
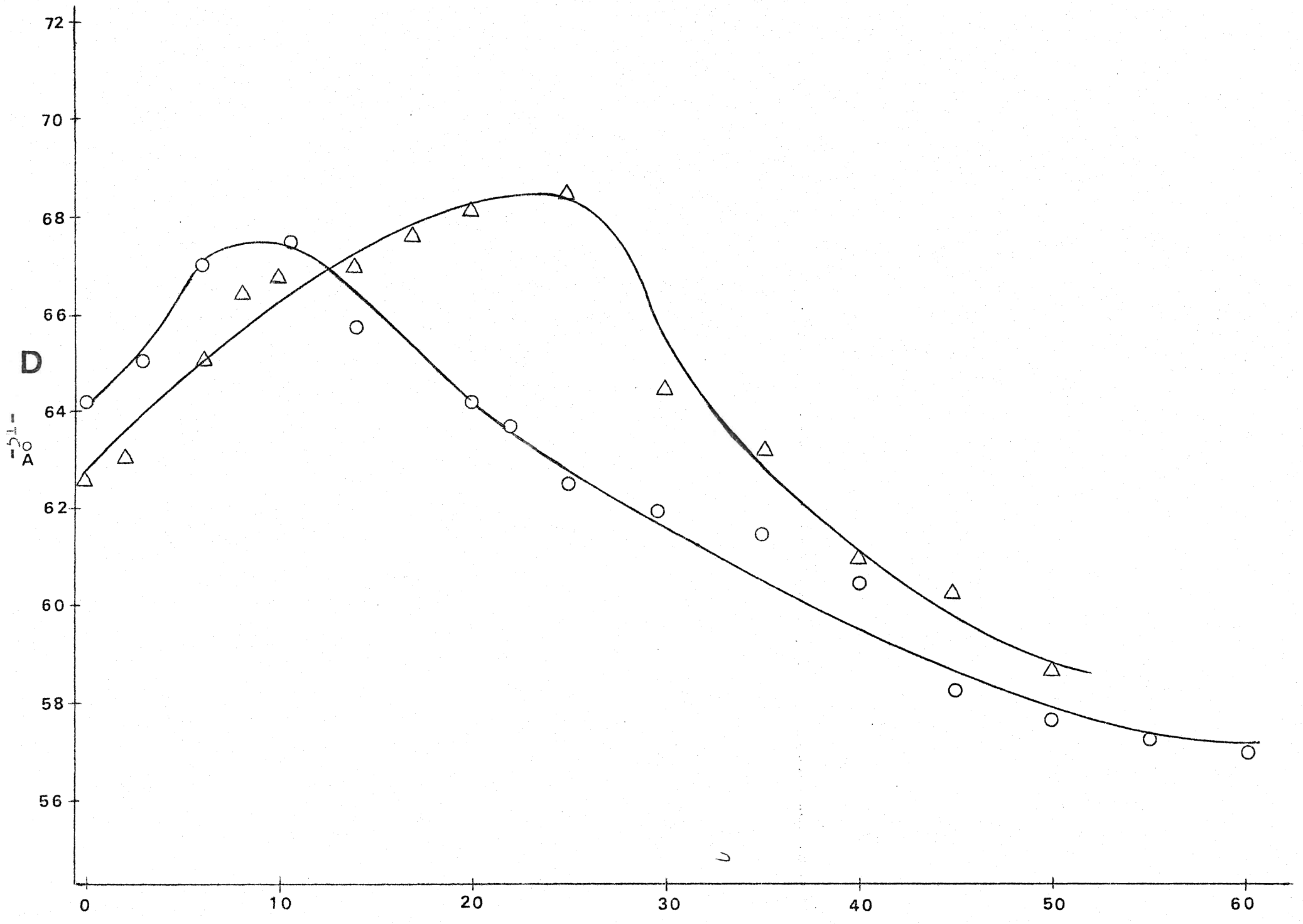

56
58
60
62
64
66
68
70
°A
D
0
10
20
30
40
50

Fig. XXI. Curves illustrating the dependence of d on the per cent sucrose for samples of "fresh" egg lecithin and "aged" egg lecithin centrifuged in sucrose solutions.

O "aged" egg lecithin centrifuged in sucrose solutions

Δ "fresh" egg lecithin centrifuged in sucrose solutions


72
70
68
66
64
62
60
58
56
D
-52-
A
0
10
20
30
40
50
60

water displayed the same lamellar repeat as control samples of egg lecithin centrifuged in distilled water.

(3) Interpretation for samples prepared gravimetrically

The data for samples prepared gravimetrically to less than excess sucrose concentration shows that until very high sucrose concentrations, d_1 does not change with added sucrose over the entire range of ϕ_1 (volume fraction of lecithin). This would indicate that any change in d, for the samples in excess is attributable to a change in d_w rather than d_1.

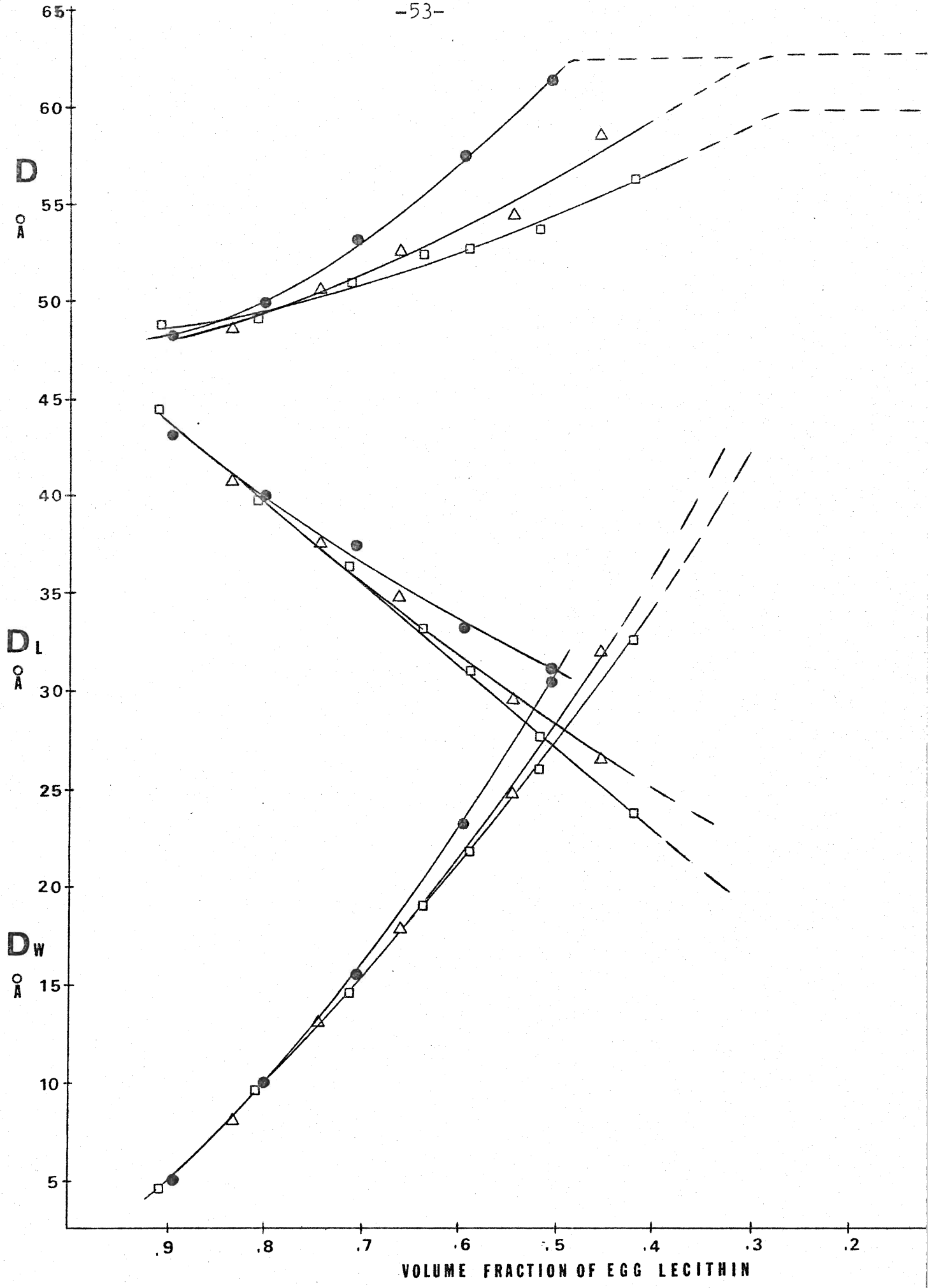
At high sucrose concentrations, d_1 appears to decrease from that of distilled water. At these concentrations crystalline sucrose appears in many samples. This could be explained by the following argument. As the sucrose concentration increases, the amount of water available, decreases. As this happens the lipid and sugar will compete for the remaining water in the samples. When the water becomes sufficiently scarce, sucrose begins to crystallize. This effect is analogous to the phenomenon called "salting out", where the addition of an electrolyte to a solution will decrease the solubility of a neutral solute, (W. J. Moore in "Physical Chemistry"). The crystallization could perhaps account for the apparent decrease in d_1. It is possible that, in the samples where d_1 decreases, there are tiny crystals of sucrose that are of insufficient density to scatter the x-ray beam appreciably. The x-ray films then would not indicate the presence of

Fig. XXII. Curves illustrating the dependence of d, d_l and d_w on the volume fraction of lipid for egg lecithin in 0, 4, and 16% dextran solutions.

● egg lecithin in distilled water (control curve)

Δ egg lecithin in 4% dextran solution

□ egg lecithin in 16% dextran solution


65
60
55
50
45
40
35
30
25
20
15
10
5
D
°A
D_L
°A
D_W
°A
.9
.8
.7
.6
.5
.4
.3
.2
VOLUME FRACTION OF EGG LECITHIN

the crystals. Since the crystals would not be in the lamellar phase, both ϕ_1 and d_1 for the lecithin lamallae would be calculated to be lower than actual.

(4) Interpretation for samples prepared by centrifugation

In the samples that have been centrifuged such that they are in excess sugar solution, the values for d are observed to rise, reach a maximum, and then decrease as the glucose or sucrose concentration increases. In order to explain this behaviour, the forces of stabilization between the lamallae of the lecithin liquid crystal can be considered. Glucose and sucrose are neutral molecules and will introduce no charge into the system. The static dielectric constant for solutions of each decrease only slightly as sugar concentration increases (Malmberg and Maryott, 1950). Addition of sugar, therefore, should have little effect on the repulsive forces between lamallae. The Van der Waals attractive forces, however, can be expected to change with added sugar, according to the predictions of Parsegian and Ninham. The key to the explanation of this change lies in the dielectric behaviour of sucrose solutions (or glucose). As indicated in Table XXIV, the refractive index and thus the high frequency dielectric susceptibility, ($\epsilon = n^2$), increases with increasing sucrose concentrations. This change should affect the Van der Waals force which is sensitive to small changes in dielectric susceptibility at high frequencies. As the dielectric susceptibility of

sucrose solution increases, it will begin to approach the dielectric susceptibility of the lipid which will be approximately the same as that of hydrocarbon. Recalling that the Van der Waals attractive force between planar layers is proportional to $(\epsilon_1 - \epsilon_2)^2$, it is clear that the attraction between lamellae will be minimal when ϵ lipid = ϵ sucrose solution. The distance between lamellae, at this sucrose concentration, would expected to be a maximum. The lamellar repeat distance d, is maximal at 25% sucrose. Therefore, ϵ_s at 25% sucrose should equal ϵ_h. (ϵ_s at 25% sucrose = n_s^2 = 1.88) Past this point ϵ_s becomes greater than ϵ_h and the Van der Waals force begins to increase. In response to this, the lamellae move closer together once more.

The optical frequency dielectric behaviour for glucose solutions as indicated in Table XXIII is almost identical to that for sucrose, (Zerban and Martin, 1944). This would account for the fact that samples centrifuged in glucose solutions behave in a manner similar to those centrifuged in sucrose.

It is not certain whether or not the concentration of the sucrose between the lipid layers is the same as the concentration in the pool for the centrifuged samples. Le Fevre et al (1968) using a system in which lecithin micelles were dissolved in an oil phase that forms an interface with sucrose solution, found that the micelles would accumulate sucrose solution internally, of higher

concentration than in the bulk phase. The lecithin in the centrifuged samples could similarily "bind" sucrose. Parsegian in a personal communication to R. P. Rand showed, by taking the derivative of the Van der Waals force with respect to the number concentration of sucrose molecules, N_s, that there was an attraction between the sugar molecules and the lipid that approximates to $(\epsilon_h - \epsilon_s) \frac{\partial \epsilon_s}{\partial N_s}$. The derivative $\frac{\partial \epsilon_s}{\partial N_s}$ is positive and approximately proportional to the molecular weight. Parsegian's estimates, however, showed that attraction would not be significant for small molecules such as sucrose. According to Parsegian's predictions, then, the lipid samples centrifuged in sucrose would not be expected to "bind" sucrose.

The experiments where sucrose, glucose and dextran samples were recentrifuged in distilled water indicates that the interactions are reversible, ie., dextran, glucose and sucrose are not complexing with the lipid in a strong interaction.

B) Experiments with Dextran

(1) Samples prepared gravimetrically

The data for the samples prepared gravimetrically for samples containing 0, 4 and 16 per cent dextran solution are given in Tables XV, XVI, and XVII respectively. This data has been illustrated in Figure XXII. The calculated values for the thickness of the lecithin leaflet,

d_1, for both 4 and 16 per cent dextran, are significantly smaller than those of the control (0% dextran solution). The minimum value of d_1 for the dextran samples, about 22 Å, is 10 Å lower than that of the control. The point of maximal swelling has moved from .5 volume fraction in the control to .35 with both 4 and 16% dextran. At 16 per cent dextran, at the point of maximal swelling, even though the aqueous layer is thicker, the absolute value of d is smaller than that of the control. The decrease in d is accounted for by the large decrease in d_1. In Figure XXII the values for d at the point of maximal swelling, indicated by the dashed lines, have been taken from the samples centrifuged in dextran solution.

Some samples were also prepared which contained 30% dextran solution. In these samples, hard glassy lumps of dry dextran were evident. Unfortunately, dry dextran is amorphous so it will not be evident in a different pattern. At concentrations lower than 30% it is impossible to tell whether or not the dextran remains separate from the lecithin lamellar phase.

(2) Samples prepared by centrifugation

The data for lecithin samples centrifuged in dextran solutions is given in Table XX and Figure XX. Up to about 5% dextran, the values for d stay constant or perhaps increase slightly. Past this point, d decreases steadily. By 40% dextran solution, d has decreased by about 8 Å to a value of 55 Å. It should be noted that the

x-ray photographs indicate that the centrifuged dextran samples were more ordered than the sucrose. The dextran samples gave up to three sharp lines on the x-ray film while the sucrose samples gave two broad lines. X-ray photographs of lecithin centrifuged in distilled water, sucrose solution, and dextran solution are shown in Figure XII.

(3) Dielectric and density data for dextran solutions

Data from the results of experiments determining the density and refractive indices of dextran solutions is given in Tables XXV and XXII. In each case, the values for dextran solutions are nearly identical to those for sucrose of the same weight per cent. (Tables XXIV and XXVI) The density data for dextran has been used to calculate d_1 for the gravimetrically prepared samples. The dielectric data (refractive indices) has been refered to in the interpretation of the results.

(4) Interpretation

a) Dielectric considerations

The results for the samples centrifuged in dextran are more difficult to explain than the results for samples centrifuged in sucrose. The refractive indices and thus, the optical frequency dielectric behaviour of dextran solutions has been shown to be nearly identical to that of sucrose solutions. As a result, the attractive forces across a dextran solution would be approximately the same as across sucrose. The dextran samples would then be

expected to behave in the same way as the sucrose samples. Since they do not, there must be some other considerations involved.

Pollack et al (1965) found that a 10% solution of clinical dextran had a static dielectric constant of 483. Wnuk (personal communication) found the dielectric increment of dextran of molecular weight 500,000 to be 10.1. This means a 10% solution would have a static dielectric constant of approximately 1000. It is generally accepted that there exists an electrostatic repulsive force between lecithin lamallae although the explicit formulation of this force remains in doubt. (Shah and Shulman, 1965, Friedenberg et al, 1966, Parsegian, 1967). Such a repulsive force will vary as the inverse of the dielectric constant of the medium between the lecithin lamallae. Since this dielectric constant increases greatly with dextran, it would be expected that the repulsive forces would decrease. The lecithin lamallae would be expected to move closer together in response to such a decrease. This effect may account for the fact that the lamellar repeat distance, d, does not increase significantly over the low range of dextran concentrations.

Another explanation for the behaviour of the dextran samples may lie in the fact that the attractive force, $(\epsilon_s - \epsilon_n) \frac{\partial \epsilon_s}{\partial N_s}$, between the lipid and dextran molecules will be significant for the high molecular weight dextran molecule. Parsegian's estimates show that

the liquid crystal should accumulate dextran from the pool such that the actual concentration between layerswould be higher than bulk. The lamellar repeat distance, d, would then be expected to begin decreasing at lower dextran concentrations than observed for the same sucrose or glucose concentrations. This is in fact, observed. Perhaps both effects are in operation.

b) Dextran-water, lecithin-water, two phase system

Another possible explanation for the behaviour of the dextran samples arises through steric considerations. A spherical dextran molecule of molecular weight 250,000 would have a radius of 200 Å, assuming a partial specific volume of dextran of .82 c.c./g. (Polymer Handbook). The lecithin bilayers are separated by an aqueous space of only about 25 Å. This is far too small to accomodate a spherical dextran molecule. Because of the hydrophilic nature of the O-H groups of the dextran, it is unlikely that the dextran molecule will penetrate into the hydrocarbon region of the bilayer. It could be then, that the dextran does not enter into the lecithin phase. If this possibility occurs then the external dextran and the lecithin will be in osmotic competition for the water. As the dextran solution becomes more concentrated, its chemical activity will increase, and water will be drawn away from the lecithin. As this happens, d_w will decrease and the lamallae will move closer together. Since d does not decrease until about 8% dextran, it would appear that

until this point the dextran solution is not sufficiently concentrated to have an effect.

This simple explanation of the results would also account for the fact that the samples centrifuged in dextran are more ordered than those centrifuged in sucrose, ie., the dextran samples are as ordered as those in distilled water simply because only water appears between the bilayers.

In the samples prepared gravimetrically with dextran, it is difficult to accept that the large decrease in d_1 over that of the control samples is real. As in the centrifuged samples there may be an equilibrated two phase system, one of lecithin and water, and one of dextran and water. In this case ϕ_1 and d_1 would be calculated to be falsely low. It would appear then, that the two phase system could be used to adequately explain the results for both the gravimetric and the centrifuged samples, and is therefore the most reasonable explanation of the results.

Addition of Sucrose to the Monocaprin Lamellar Phase

The results from the monocaprin sucrose study given in Figure XXIII and Table XIII, are rather inconclusive. There appears to be little change in d and d_1 with 40% sucrose as compared to the control. These results are equivalent to those with egg lecithin, and are to be expected. Unfortunately, monocaprin goes into a dispersion phase in excess water so the centrifuged samples

which would be the most informative cannot be prepared (Larsson, 1967).

General Remarks

It appears as if the electrodynamic theories discussed in part I provide satisfactory explanations for many of the results observed in these experiments. As expected, d_w increases upon addition of a charged lipid to the lecithin bilayer. Glucose and sucrose solutions which have similar dielectric properties, give similar results when mixed with lecithin. The rise and fall of the lamellar repeat distance d in the centrifuged samples, are perhaps the most significant of the results and are adequately explained by the theory. These experiments then, lend considerable support to the theoretical work of Parsegian and Ninham.

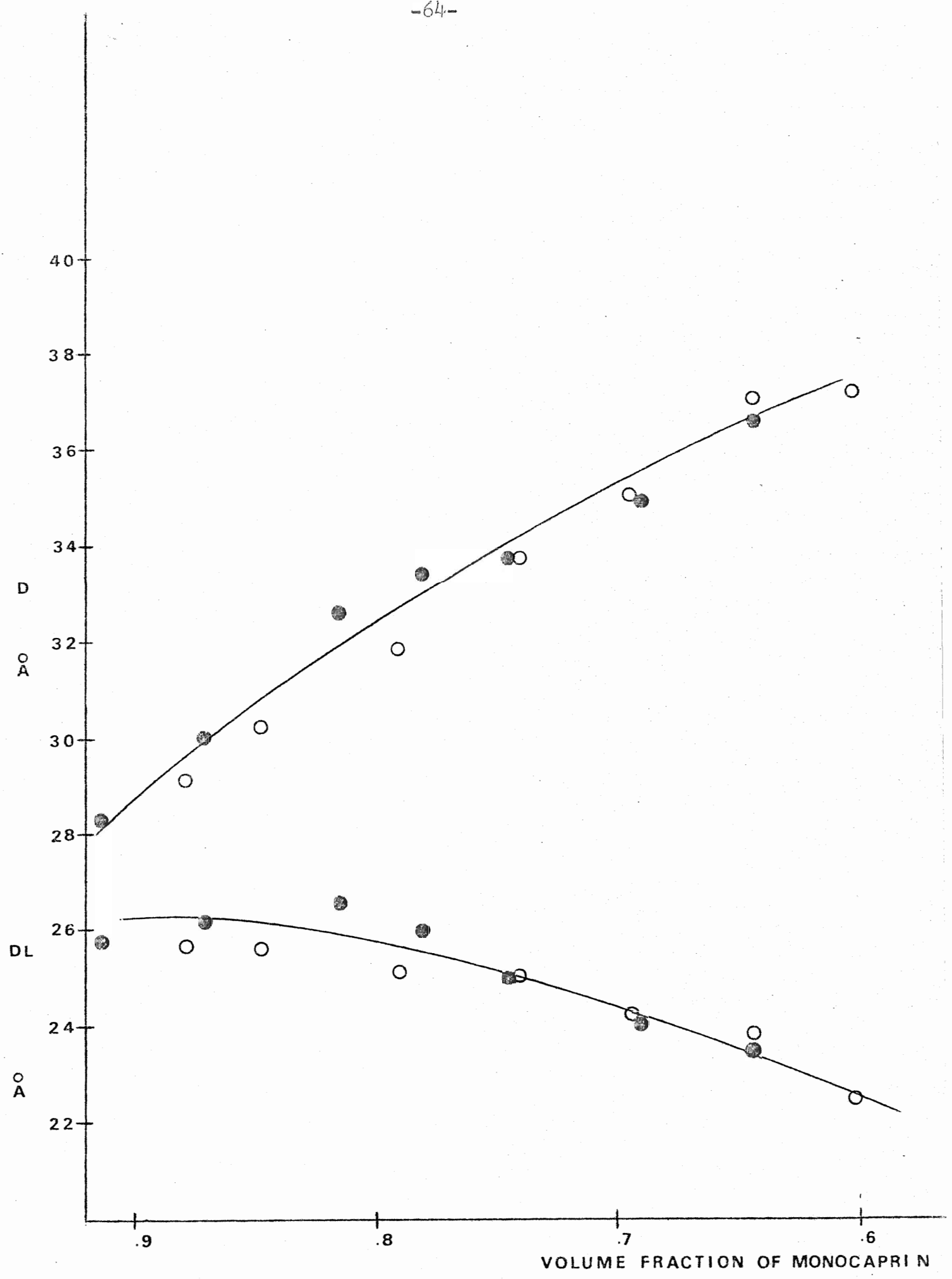
Dextran solutions have different dielectric properties than glucose or sucrose and give different results when mixed with lecithin. It seems likely, however, that the dextran due to its large size, is not entering into the lecithin phase. Further experimentation with lower molecular weight dextran molecules might provide some illumination in this area.

There is some likelihood that the same forces that act in these systems will be determining factors in the consideration of surface contact phenomena in real membranes. Worthington and Blaurock (1969) reported that a

Fig. XXIII Curves illustrating the dependence of d and d_1 on the volume fraction of monoglyceride for monocaprin in distilled water and in 40% sucrose solution.

● monocaprin in 40% sucrose solution

o monocaprin in 0% sucrose solution (control curve)


40
38
36
34
D
32
°
A
30
28
26
DL
24
°
A
22
.9
.8
.7
.6
VOLUME FRACTION OF MONOCAPRIN

.24 M sucrose solution had the effect of increasing the repeat distance membraneous layers in the myelin sheath from 252 Å (in distilled water) to 388 Å. This is one result that could easily be explained by using the theories considered here.

Other questions that arise in Part I are, why the sucrose and glucose solutions appear to have a disordering effect on lecithin samples, and what factors are important in effecting the thickness of the lipid bilayers. It appears that neither addition of sucrose nor addition of charged lipid has any effect on the bilayer thickness. Gotleib and Eanes (1972), on the other hand, found that certain electrolytes were effective in changing the bilayer thickness. Membrane fuctions such as permeability properties and enzymatic activities would be sensitive to membrane thickness, thus such considerations are of biological importance.

Table I

Pig-liver lecithin control (0% CTAB)

T = 25°C

% lipid by weight	d in Å	d_l in Å	d_w in Å
89.9%	44.7	40.2	4.5
79.7%	45.8	36.6	9.2
68.5%	50.2	34.4	15.8
59.7%	52.6	31.4	21.2
50.3%	60.3	30.3	30.0
39.8%	64.1		
29.8%	64-70*		
20.0%	68.8		

* The samples that show a spacing range are poorly equilibrated. The lines on the x-ray film either display a gradient or are fuzzy, consequently the spacing must be estimated.

Table II

Pig-liver lecithin - 5 mole % CTAB

T = 25°C

% lipid by weight	d in Å	d_l in Å	d_w in Å
90.0%	42.4	38.2	4.2
79.5%	44.5	35.4	9.1
70.3%	47.2	33.2	14.0
59.8%	51.0	30.5	20.5
47.3%	61.3	29.0	32.3
36.8%	73-79	27-29	46-50

Table III

Samples in excess water varying % CTAB

T = 25° C

mole % CTAB	% lipid by weight	d in Å
7.0	38.1	64-77
9.0	40.1	74-81
11.0	33.2	97-117

Table IV

Structural data for egg lecithin in distilled water

T = 25° C

% egg lecithin by weight	d in Å	d_l in Å	d_w in Å
94.6	49.0	46.3	3.7
90.5	49.3	44.6	4.7
80.0	50.5	40.4	10.1
70.4	54.1	38.0	16.1
64.0	57.8	37.0	20.8
60.1	59.5	35.8	23.7
56.0	62.5	35.0	27.5
50.0	62.5		
40.0	62.5		

Table V

Structural data for 70% egg lecithin in varying % sucrose solution

T = 25°C

weight % egg lecithin in sample	weight % sucrose solution	d in Å	d_1 in Å
69.4	0	53.6	37.2
70.4	5	53.5	73.9
70.3	10	54.4	38.6
69.1	15	55.0	38.6
69.3	20	55.0	39.0
70.3	25	52.7	38.2
70.0	30	53.8	39.0
69.6	35	53.7	39.0
70.4	40	53.0	39.1
70.0	45	52.5	38.7
69.8	50	50.7	37.6
69.4	55	49.5	36.1
70.0	*60	48.5	

Table VI

Structural data for .72 volume fraction egg lecithin in varying % sucrose solution

T = 25°C

volume fraction of egg lecithin in sample	weight % sucrose solution	d in Å	d_1 in Å
.719	0	54.0	38.8
.721	2	53.7	38.7
.721	5	54.3	38.9
.720	7	54.7	39.4
.717	9	54.4	39.0
.722	12	54.5	39.4
.718	15	55.4	39.8
.725	20	55.0	39.9
.722	25	54.2	39.1
.720	30	55.2	39.7
.716	35	55.0	39.4
.723	40	55.5	40.2
.719	45	52-55	37.4-39.5
.720	50	51.3	36.9
	*55	52.0	
	*60	53.7	
	*65	53.8	

* In these samples crystalline sucrose appeared. This showed up as a series of sharp reflections on the x-ray film.

Table VII

Control samples (0% sucrose) for egg lecithin in 30% sucrose solution

T = 25°C

volume fraction of egg lecithin	d in Å	d_1 in Å
.880	49.0	43.1
.843	50.5	42.6
.788	51.0	40.2
.693	53.3	36.9
.644	55.7	35.8

Table VIII

Structural data for egg lecithin in 30% sucrose solution

T = 25°C

volume fraction of egg lecithin	d in Å	d_1 in Å
.922	48.5	44.7
.875	49.6	43.4
.833	51.0	42.5
.786	51.7	40.6
.736	53.7	39.5
.674	57.0	38.4

Table IX

Structural data for control samples (egg lecithin in 0% sucrose) for the 22%, 40%, and 50% sucrose series

T = 25° C

volume fraction of egg lecithin	d in Å	d_1 in Å
*.897	50.2	44.9
.896	49.6	44.4
.845	49.3	41.7
.805	50.7	40.8
.769	51.4	39.6
*.757	52.5	39.7
.713	53.9	38.4
.675	55.7	37.8
.626	58.3	36.5
*.603	60.2	36.3
.444	62.7	

* The starred control samples were prepared after the 22, 40, and 50% series, while the others were prepared before.

Table X

Structural data for the egg lecithin in 22% sucrose solution

T = 25° C

volume fraction of egg lecithin	d in Å	d_1 in Å
.908	49.2	44.7
.878	49.1	43.1
.846	49.8	42.2
.813	50.4	41.0
.774	52.0	40.2
.747	53.1	39.6
.699	55.0	38.4
.671	56.4	37.8
.635	59.7	37.9
.604	60.6	36.6
.546	65.6	35.8

Table XI

Structural data for egg lecithin in 40% sucrose solution

T = 25° C

volume fraction of egg lecithin	d in Å	d_1 in Å
.921	50.0	46.1
.893	50.7	45.2
.794	52.0	41.2
.763	53.7	41.0
.717	55.4	39.8
.687	57.0	39.2
.618	58.8	36.4
.578	60.6	

Table XII

Structural data for egg lecithin in 50% sucrose solution

T = 25° C

volume fraction of egg lecithin	d in Å	d_1 in Å
.924	*44.1	
.892	*47.3	
.852	*49.7	
.829	*50.1	
.793	*51.3	
.752	53.7	40.3
.713	53.9	38.5
.669	54.8	36.7
.631	56.3	35.5
.592	57.4	34.0

* In these samples crystalline sucrose appeared. This showed up as a series of sharp lines on the x-ray film.

Table XIII

Structural data for the monocaprin control samples (0% sucrose)

T = 25° C

volume fraction of monocaprin	d in Å	d_1 in Å
.878	29.1	25.6
.847	30.2	25.6
.790	31.8	25.1
.741	33.7	25.0
.692	35.0	24.2
.643	37.0	23.8
.602	37.3	22.4

Table XIV

Structural data for the monocaprin in 40% sucrose solution

T = 25° C

volume fraction of monocaprin	d in Å	d_1 in Å
.913	28.3	25.8
.875	30.0	26.2
.815	32.6	26.6
.780	33.4	26.0
.742	33.8	25.0
.690	35.0	24.1
.643	36.6	23.5

Table XV

Structural data for the control samples (0% dextran solution) for egg lecithin in dextran solution

T = 25° C

volume fraction of egg lecithin	d in Å	d_l in Å	d_w in Å
.896	48.3	43.3	5.0
.802	50.0	40.1	9.9
.707	53.2	37.6	15.6
.596	57.4	34.2	23.2
.505	61.0-62.0	30.8-31.3	30.2-30.7

Table XVI

Structural data for egg lecithin in 4% dextran solution (by weight)

T = 25° C

volume fraction of egg lecithin	d in Å	d_w in Å	d_l in Å
.837	48.7	7.9	40.8
.745	50.6	13.0	37.6
.662	52.6	17.8	34.8
.544	54.4	24.8	29.6
.454	58.4	31.9	26.5

Table XVII

Structural data for egg lecithin in 16% dextran solution

T = 25° C

volume fraction of egg lecithin	d in Å	d_w in Å	d_l in Å
.908	48.8	4.4	44.4
.808	49.3	9.5	39.8
.713	50.9	14.6	36.3
.637	52.2	19.0	33.2
.589	52.7	21.7	31.0
.516	53.7	26.0	27.7
.420	56.3	32.6	23.7

Table XVIII

Structural data for egg lecithin centrifuged in sucrose solutions T = 25° C

% dextran by weight	d in Å
0	62.5
2.0	63.0
6.0	65.0
8.0	66.4
10.0	66.7
12.0	66.6
14.0	66.9
17.0	67.6
20.0	68.1
25.0	68.4
30.0	64.5
35.0	63.2
40.0	61.0
45.0	60.3
50.0	58.7

Table XIX

Structural data for egg lecithin centrifuged in glucose β D+ (dextrose) solutions T = 25° C

% β D+ (dextrose) glucose by weight	d in Å
0	63.0
5.0	64.1
10.0	64.8
15.0	65.4
20.0	67.0
22.0	68.0
25.0	64.1
30.0	61.8
35.0	60.0
40.0	58.7

Table XX

Structural data for egg lecithin centrifuged in dextran solutions T = 25°C

% dextran by weight	d in Å
0	62.1
2	62.2
3	62.8
5	62.4
6	61.8
8	61.9
12	60.4
17	59.4
20	58.7
25	57.8
30	56.7
35	56.2
40	55.0

Table XXI

Structural data for "aged" egg lecithin centrifuged in sucrose solutions T = 25°C

% sucrose by weight	d in Å
0	64.2
3.0	65.0
6.0	67.0
10.0	67.5
14.0	65.7
18.0	65.8
20.0	64.2
22.0	63.7
25.0	62.5
30.0	62.0
35.0	61.6
40.0	60.6
45.0	58.3
50.0	57.7
55.0	57.3
60.0	57.1

Table XXII

Refractive indices of dextran solutions
(molecular weight 200,000 - 275,000)
measured with an Abbe refractometer T = 24° C

% dextran by weight	n_D measured (corrected value)
0	1.333
2	1.334
4	1.338
8	1.344
12	1.350
16	1.356
20	1.363
25	1.372
30	1.381

Table XXIII

Refractive indices of dextrose, (β D+ glucose), solutions

T = 20°C

(Zerban and Martin, p.144)

% dextrose by weight in air	n_D observed
1.997	1.33585
4.001	1.33880
4.984	1.34026
5.995	1.34169
7.873	1.34451
10.025	1.34780
11.998	1.35078
15.033	1.35556
19.961	1.36353
25.028	1.37198
30.004	1.38056
35.083	1.38995
40.191	1.39911
45.290	1.40889
50.246	1.41873
55.237	1.42901
60.452	1.44013
62.652	1.44503
65.746	1.45183
71.036	1.46397
75.615	1.47478
77.022	1.47816
80.362	1.48632

Table XXIV

Indices of Refraction of aqueous sucrose solutions

T = 25° C

(Handbook of Physics and Chemistry)

% sucrose	index of refraction
0	1.3330
5.0	1.3413
10.0	1.3474
15.0	1.3551
20.0	1.3632
25.0	1.3716
30.0	1.3804
35.0	1.3894
40.0	1.3989
45.0	1.4088
50.0	1.4192
55.0	1.4298

Table XXV

Densities of aquecus dextran solutions

T = 25°C

% dextran by weight	density g/cc
4%	1.0135
16%	1.0614
30%	1.1225

Table XXIV

Densities of aqueous sucrose solutions

T = 25°C

% sucrose by weight	density g/cc
0	.99699
5	1.01654
10	1.03687
15	1.05916
20	1.08096
25	1.10185
30	1.12510
35	1.14940
40	1.17449
45	1.20038
50	1.22732
55	1.25505
60	1.28384

Part 2

INTRODUCTION

It is generally accepted that the interactions between proteins and lipids are important to membrane studies. Many investigators have studied these interactions by using acidic lipids and basic proteins (Das et al, 1962, Braun and Rodin, 1969, Leslie and Chapman, 1969, Hart et al, 1969). The interactions in these cases are primarily ionic and are determined by the basic groups on the proteins and by the electrostatic field at the lipid - water interface. Gulik-Kryzwicki et al (1969) and Rand (1971) have shown by studying ordered precipitates by x-ray diffraction that hydrophobic interactions can occur between acidic phospholipid and basic protein. Rand (1971) and Rand and Sen Gupta (1972) have studied in detail how the structure of two lipid protein systems change as the net charge of the system changes. Other authors have investigated structures formed between phospholipids and synthetic proteins. Giannoni, Padden and Roe (1971) have carried out studies using x-ray diffraction on precipitates of poly-l-tyrosine and lecithin. They discovered that these components combined in a stoichiometric quantities to form a lamellar complex. In this case the protein is uncharged so ionic binding forces are apparently not involved.

Hammes and Schullery (1970) studied, by means of circular dichroism, optical rotary dispersion, electron microscopy, and nuclear magnetic resonance, complexes formed between poly-l-lysine, phosphatidyl serine and lecithin, and poly-l-ornithine and phosphatidyl serine. The results indicated that the synthetic proteins changed from the random coil configuration in solution to the helical form in the complex with the phospholipid.

Plan of Part 2

In this study, phosphatidyl inositol, (PI), and mixtures of egg lecithin and phosphatidyl inositol have been added to solutions of a synthetic protein, poly-l-lysine. The resulting precipitates have been analyzed by x-ray diffraction. It was hoped that structures formed would be lamellar arrangements of lipid bilayers coated with protein. Such structures could be used as a model for the study of interactions between membranes of the Davson-Danielli construction.

MATERIALS AND METHODS

In this investigation, the chloride salt of poly-l-lysine of molecular weight 188,000 was used (Sigma Chemicals). The phosphatidyl inositol was a generous gift from Dr. Tinker and the egg lecithin was extracted in the laboratory.

(1) Experiments with poly-l-lysine and phophatidyl inositol

In the first experiment phosphatidyl inositol (PI) was added to protein solutions of varying concentrations. Seven, 7.0 ml solutions of the following concentrations 1 mg/ml, .05 mg/ml, .083 mg/ml, .0415 mg/ml, .01 mg/ml, .005 mg/ml, and .001 mg/ml were prepared. To each solution 1 ml of 5.0 mg/ml of PI, suspended in distilled water, was added dropwise accompanied by vigorous stirring. These were centrifuged at 160 thousand g for two hours. The supernatant was removed, distilled water was added, and the tubes were recentrifuged. The precipitates were mounted in sample holders and the x-ray pictures of them were taken.

(2) Experiments with mixtures of lecithin, PI, and poly-l-lysine

In these experiments mixtures of lecithin and PI were added to excess protein solution, ie., more than

sufficient for binding to the lipid. In a series of conical flasks, chloroform solutions of lecithin and PI were mixed in a way to give 5 mg. of lipid in each, with varying weight ratios of PI and lecithin. The weight ratios of lecithin to PI prepared were 100/0, 95/5, 90/10, 85/15, 80/20, 75/25, 70/30, 65/35, 60/40, 55/45. and 50/50. These mixtures were evaporated to dryness using a rotary evaporator and finally dried *in vacuo*. Each mixture was then suspended in 2 ml. of distilled water, and added dropwise, accompanied by vigorous stirring, to tubes containing 6.5 mls of 1mg/ml of poly-l-lysine. The x-ray samples of the precipitates were prepared in the usual manner.

Chemical analysis of the precipitates

(1) Water content

The precipitates were dried successively under a stream of nitrogen to remove excess water. The x-ray pictures were retaken after each drying. When the lamellar spacing just detectably changed dimension, the precipitates were weighed, completely dried by lyophilising overnight and reweighed to determine the water content.

(2) Lipid content

Phospholipid content of the precipitates was determined by analysing the phosphorus content of the samples. The lyophilised samples and a series of standards were digested with a mixture of 5 ml. of perchloric acid, 5 ml. nitric acid, and 5 ml. distilled water for 2 hours

under gentle heat. 50 ml of water was added and the mixtures were boiled until the volume was reduced to about 15 ml. The solutions were cooled and volumes adjusted. The phophorus content was determined spectrophotometrically with a Bausch and Lomb spectronic 20 according to the method of Chen et al., (1956). The phosphorus standard curve is shown in figure XXIV.

(3) Protein content

Attempts were made to determine the protein content independently by finding the amount left in the supernatant after precipitation. Folin analysis, the biuret method, and viscosity measurements were attempted. In the Folin analysis it appeared that anomalous light scattering by the protein in the reagent solution invalidated the spectrophotometric readings. To determine concentration of the protein by viscosity, the fact that viscosity is dependent on protein concentration, was used. A standard curve of viscosity of poly-l-lysine versus concentration was constructed and the viscosity of the supernatant solutions was compared against this. Unfortunately the viscosity of the solutions proved to be extremely sensitive to salt concentration (Fig. XXV) Since the concentration of salt in the supernatant was unknown, the method was abandoned. The biuret method on the supernatant proved of limited success. The analysis was not sensitive enough to accurately detect the small amount of protein bound.

Fig. XXIV. Standard curve for phosphorus determination. A plot of optical density (820 nm) versus phosphorus concentration in μg/ml.

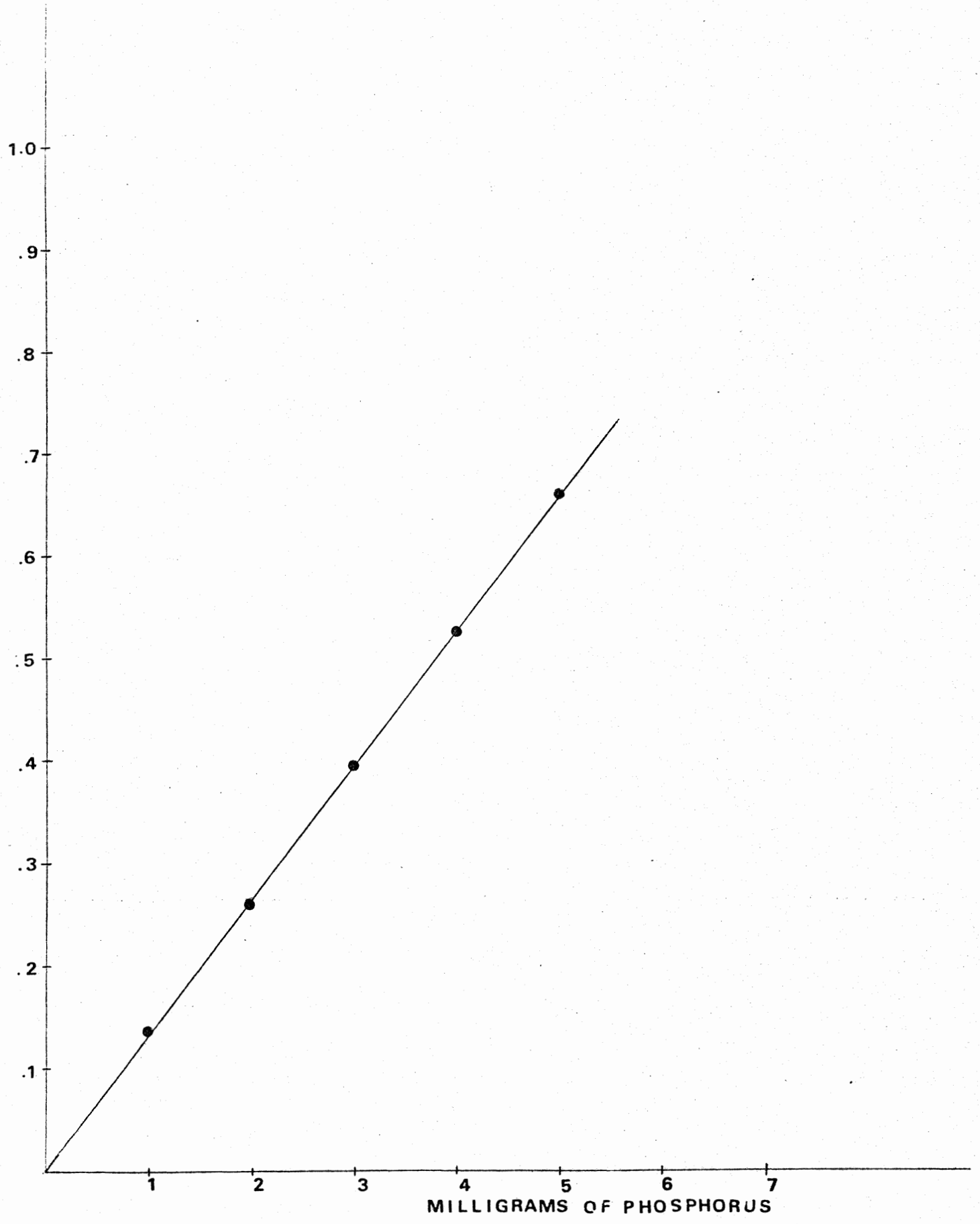
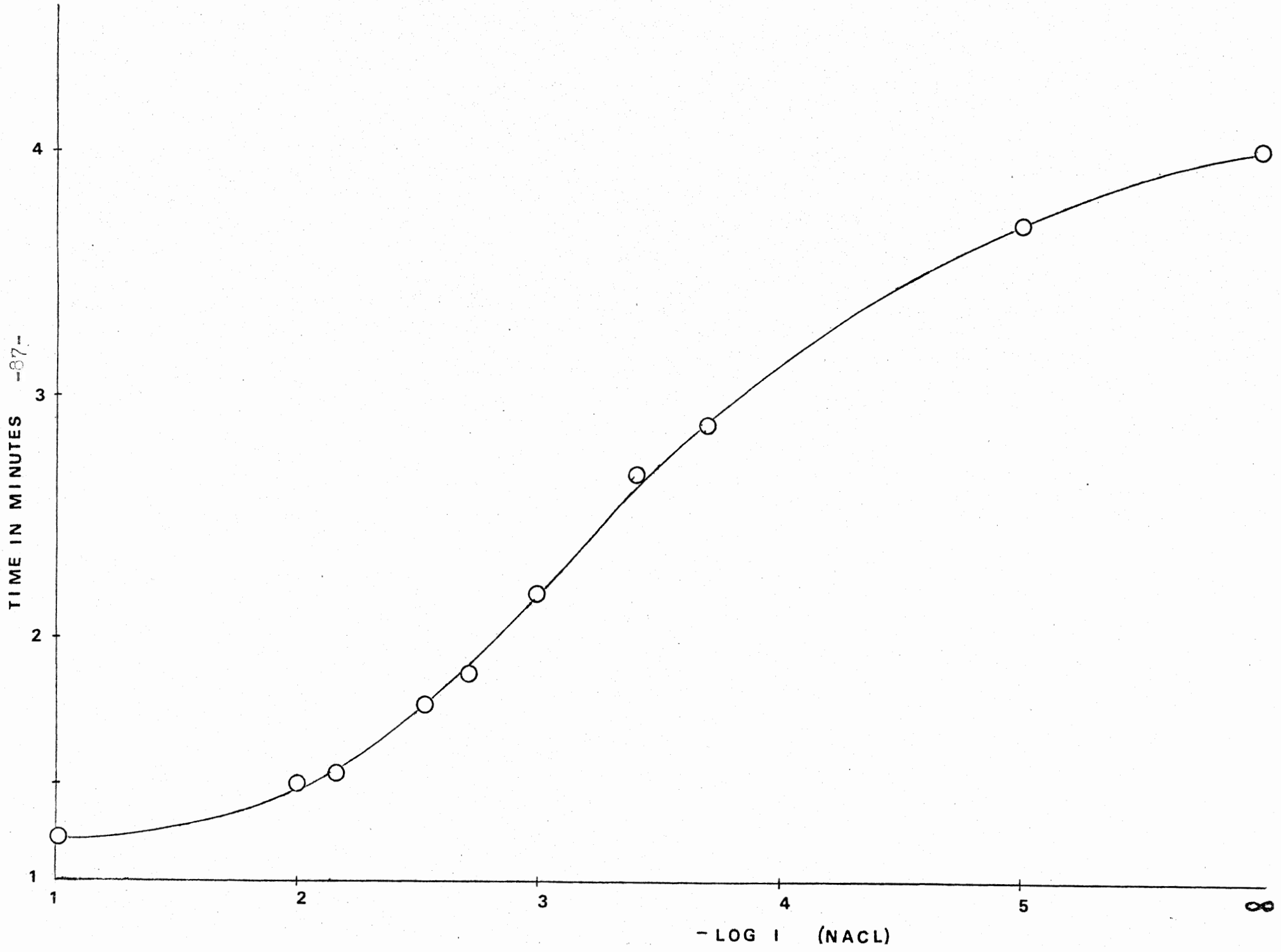

1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
1
2
3
4
5
6
7
MILLIGRAMS OF PHOSPHORUS

Fig. XXV. Curve illustrating the dependence of viscosity (represented by time of flow through an Oswald viscometer) of a .5 gm/ml poly-l-lysine solution, on the ionic strength of the solution.


TIME IN MINUTES
4
3
2
1
1
2
3
4
5
∞
- LOG I (NACL)

As a result of these difficulties the protein content was determined by difference.

RESULTS AND INTERPRETATIONS

(1) Poly-l-lysine, phosphatidyl inositol samples

In the first experiment the amount of poly-l-lysine available to the phosphatidyl inositol is progressively decreased. The precipitates for the 1 mg/ml and .05 mg/ml protein solutions appeared to be of the same size and consistency. In the others, after centrifugation, much of the lipid remained unbound forming a phase separate from the precipitate. The size of the precipitate decreased as the initial protein concentration decreased. The spacing between reflections and the intensities on the x-ray photograph are identical for all the samples indicating the structure of the precipitates is the same for each. These results indicate that poly-l-lysine and phosphatidyl inositol appear to be complexing in a stiochiometric ratio that is independent of the relative concentration of lipid and protein in the supernatant.

An independent study by R. P. Rand in this laboratory showed that phosphatidyl inositol swells infinitely in the presence of excess water. This explains why there is no diffraction from the unbound lipid on the x-ray film.

A poly-l-lysine, phosphatidyl inositol precipitate

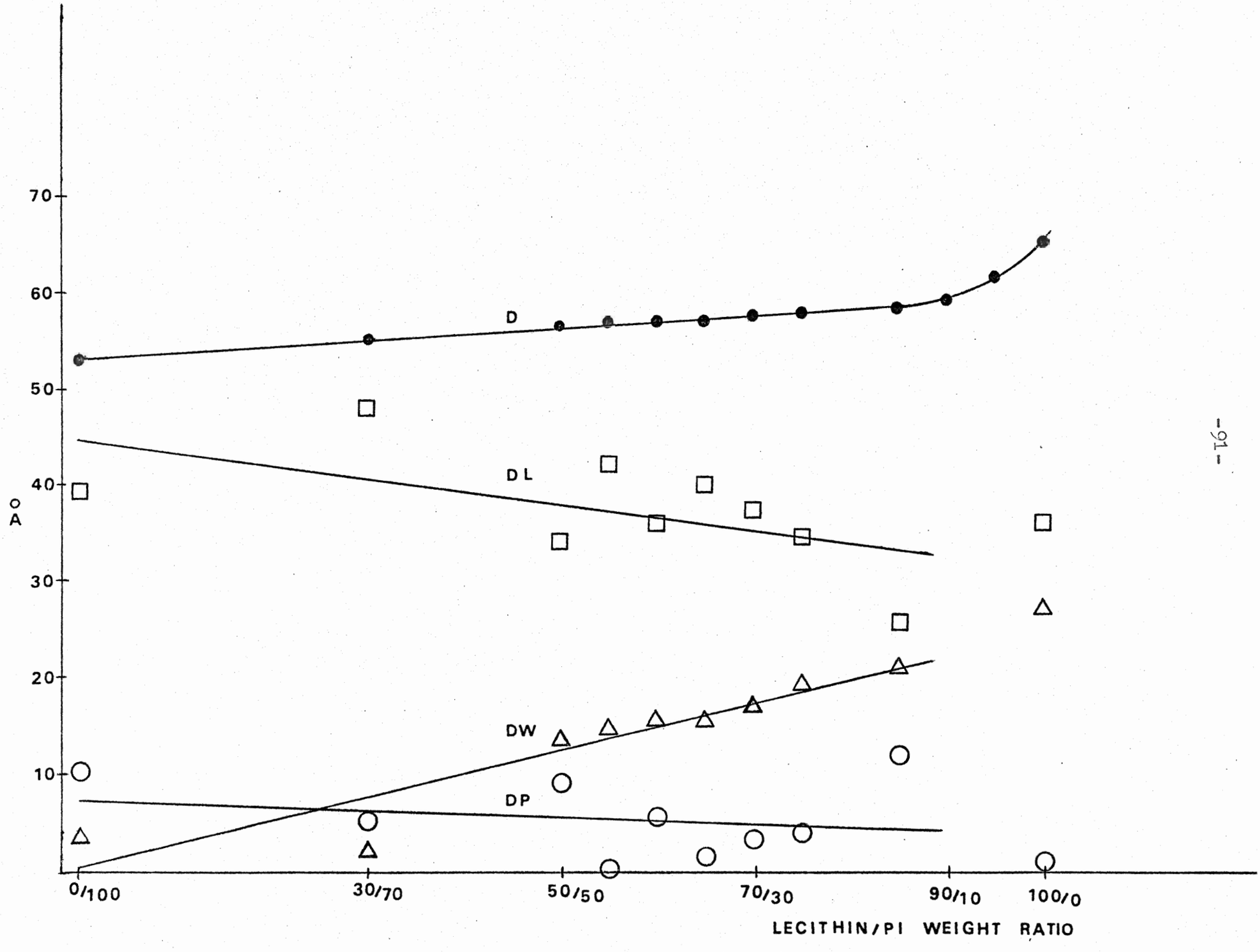
was analyzed for protein, water, and lipid content. The precipitate did not change weight upon lyophilising so it was concluded that any water was not forming a continuous discrete layer but was bound into the structure. The phosphorus analysis gave a value for ϕ_l of .87. The lipid bilayer thickness d_l is then 46 Å. ϕ_p is .13 and d_p is 7 Å. This result agreed favourably with experiments carried out at a later date.

(2) Poly-l-lysine, phosphatidyl inositol, lecithin samples

In the second experiment the solutions were prepared such that the protein was always in excess, ie., present in solution after precipitation. With high PI content, the precipitates appeared macroscopically, dry and compact. They became progressively more fluid as the lecithin content was increased. Between the L/PI weight ratios of 0/1 and 85/15, the precipitates are quite ordered as indicated by the presence of up to six sharp reflections on the x-ray photographs. Between 85/15 and 100/0, L/PI weight ratio, the precipitates became extremely fluid and were quite disordered as shown by the decrease in number and the broadening of the x-ray reflections.

The structural parameters for the precipitates are given in Table XXVIII and Figure XXVI . There is little scatter in the values for the repeat distance d and in the values for ϕ_w and d_w. The values for ϕ_l and d_l are more scattered. This is due to errors in the phosphorous

Fig. XXVI. Curves illustrating the dependence of the structural parameters, d, d_l, d_p and d_w on the lecithin/PI weight ratio for precipitates containing lecithin, phosphatidyl inositol and poly-l-lysine.


70
60
50
40
30
20
10
Å
D
DL
DW
DP
0/100
30/70
50/50
70/30
90/10
100/0
LECITHIN/PI WEIGHT RATIO

analysis of the samples. The values for ϕ_p and d_p were obtained by difference, so reflect the scatter in the values for ϕ_l and d_l.

The values for d increase linearly from 53 Å, at 0/1 L/PI ratio, to 58.5 Å, at 85/15 L/PI ratio. Between 85/15 and 100/0 L/PI ratio, d rises more sharply. Between 0/1 and 85/15 L/PI ratio, d_w increases linearly from .5 Å to 21 Å. In order to obtain an indication of the trend that the values of d_l and d_p display with changing lecithin content in the precipitates, the data for each have been fitted to a straight line by the method of least squares analysis. The values for d_p appear to decrease only slightly from 8 Å to 4.5 Å as the lecithin content increases from 0 to 85%. d_l appears to decrease from 45 Å to 38 Å over the same range. Since the samples with weight ratios of 90/10 and 95/5 L/PI were too fluid to be analyzed, the lipid, protein and water content could not be found.

The results show that d_l is exactly the same as for pure PI in water, ie., d_l for a relatively dry sample of pure PI is 45 Å and decreases as the water content increases (R. P. Rand, unpublished results). This indicates that the protein does not hydrophobically bind to the lipid but remains entirely in the aqueous space. The binding of the protein would then be strictly ionic in nature. It should be mentioned that the d_w, d_p, and d_l values quoted are hypothetical. The calculations for

these values were made assuming the lipid, protein, and water each formed a single continuous layer. The lipid seems to form a discrete layer but undoubtedly there will be considerable water in the protein layer in order to solubilize the charges. In this respect it may be more realistic to refer to a mixed protein and water layer of thickness $d_p + d_w$.

Charge Density in Precipitates

From the least squares analysis for the thickness of the protein layer, d_p and the lipid layer d_l, the number of poly-l-lysine molecule per PI residue can be calculated. The amount of bilayer surface area for every PI molecule, the amount available to every lysine residue, and the net surface charge density can also be calculated. This data is given in Table XXIX. In the pure PI, poly-l-lysine precipitate there are 1.4 lysine residues per PI while for the ratio 85/15 lecithin/PI there are 7.6 residues per PI. There are about 1140 lysine residues per protein molecule so the number of PI molecules per poly-l-lysine molecules decreases from about 810 to 150 as the lecithin content increases. The net charge per unit bilayer surface area remains constant after 50% lecithin, at about .021 charges per square angstrom. (Assuming that one PI molecule ionically binds one lysine residue, the net charge is the number of unbound residues).

It would appear then, that as the lecithin content

increases and the PI molecules become more widely spaced in the bilayer, the amount of protein bound decreases slightly, and the net charge density remains constant. The water content, no doubt increases, in order to solubilize the increasing number of unbound lysine residues. As the lecithin content increases, the lamellar repeat distance, d, increases by 5.5 Å. This small increase can be attributed to an increase in the water content rather than an increase in charge density. When the lecithin content of the lipid becomes higher than 85% the samples become disordered and d increases to that of pure lecithin. In this region it seems that there are insufficient PI molecules to bind the protein tightly so that the lipid layers become free to swell.

The configuration of the poly-l-lysine molecules on the bilayer surface cannot be determined by the methods used here. Tinker et al. (private communication) found that optical rotary dispersion and circular dichroism measurements indicated that poly-l-lysine changed from random coil in solution to α helical upon complex formation with PI. Hammes and Schullory observed the same result when poly-l-lysine was complexed with phosphatidyl serine. The diameter of the backbone chain of the αhelix is 6 Å (in Biochemistry by A. L. Lehninger). The lysine residues will extend 7.5 Å from the helix. Since the protein plus water layer ($d_p + d_w$) is 8.5 Å for the pure PI precipitate, it is quite possible that a single

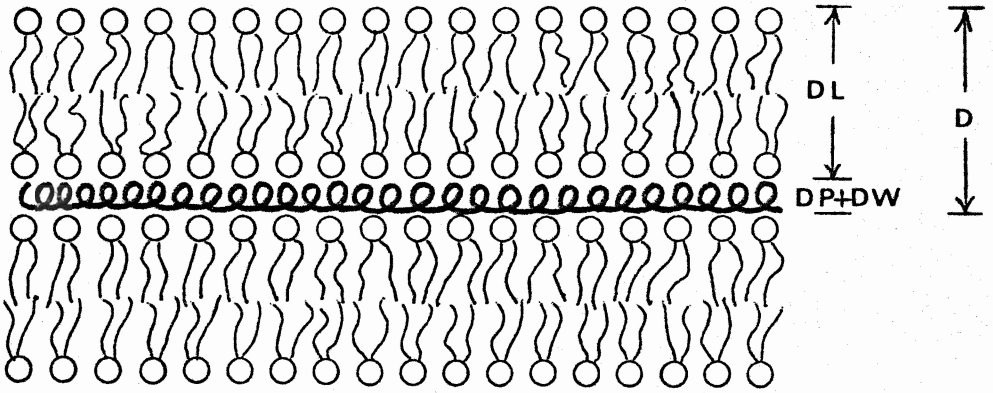
helical poly-l-lysine molecule will be bound to adjacent bilayers. The lysine residues are of sufficient length that they could penetrate into the head group region of the bilayer ionically binding with the PI molecules. As the lecithin and water content in the precipiates increase randomly coiled sections of the protein could bridge α helical sections bound to opposite lipid bilayers. This is illustrated in Figure XXVII. The binding of the protein to both sides of the aqueous layer would prevent the lamellae from swelling until this binding breaks down. This break down in binding would then be expected to occur in the samples with lecithin content of the lipid greater than 85%.

It should be emphasized that the model proposed here, for the structure of the precipitates, is speculative but conforms to the experimental data. The binding of the protein into the complex seems to be of a strictly ionic nature. The proposed structure is, as expected, similar to that of the Davson-Danielli model, ie. a lipid bilayer coated by an ionically bound layer of protein. Unfortunately, this structure is not suitable for the study of force balancing since the large protein molecule binds adjacent lipid layers together, thus prohibiting any swelling. In order to carry out such studies, another lipid-protein system must be sought.

Fig. XXVII. Diagrammatic representation of the hypothetical arrangement of lipid protein and water in two different precipitates containing lecithin, phosphatidyl inositol and poly-l-lysine.

The top illustration is a representation of a precipitate containing no lecithin.

The bottom illustration is a representation of a precipitate with a lecithin/PI weight ratio of 70/30.


DL
D
DP+DW

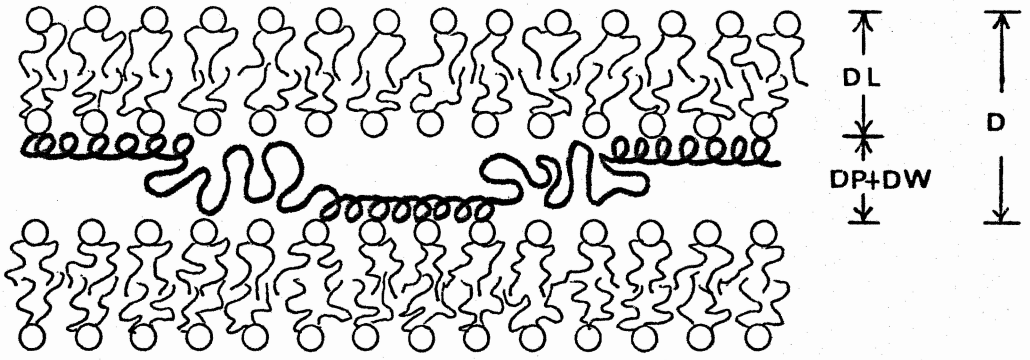

DL
D
DP+DW

Table XXVIII

Structural parameters of the lecithin PI, poly-l-lysine precipitates

L/PI weight ratio	ϕ_l	ϕ_p	ϕ_w	d(Å)	d_l(Å)	d_p(Å)	d_w(Å)
0/1	.73	.19	.08	53.0	39.3	10.2	3.5
30/70	.87	.09	.04	55.0	47.8	5.0	2.2
50/50	.62	.17	.21	56.5	33.8	9.3	13.4
60/40	.66	.10	.24	57.0	36.0	5.5	15.5
65/35	.74	.03	.23	57.0	39.9	1.6	15.5
70/30	.68	.06	.26	57.5	37.4	3.3	16.8
75/25	.60	.07	.33	57.8	34.5	4.0	19.3
85/15	.46	.21	.33	58.5	25.8	11.8	20.9
90/10	-	-	-	-			
95/5	-	-	-	-			
100/0	.56	.03	.41	65.0	36.2	1.7	27.1

Table XXIX

Charge densities

L/PI weight ratio	no. lysine residues per PI (η)	surface area per PI molecule in sq. Å	surface area available per lysine residue in sq. Å	net charge per unit area (σ) charges/sq Å
0/1	1.4	33	24	.012
30/70	1.9	53	28	.017
50/50	2.7	79	32	.022
55/45	2.9	89	33	.021
60/40	3.2	102	34	.022
65/35	3.4	119	34	.020
70/30	4.0	141	36	.021
75/25	4.7	173	37	.021
85/15	7.6	300	39	.022

Formulae for calculations:

$$A_{lysine} = \frac{\text{residue mol. weight}}{6.02 \times 10^{23}} \times \frac{\sqrt{lysine}}{d_p}$$

$\sqrt{lysine}$ - partial specific volume poly-l-lysine = .82 (Polymer Handbook)

$$A_{PI} = \frac{\text{mol. weight PI}}{6.02 \times 10^{23}} \times \frac{\sqrt{lipid}}{\%PI} \qquad (\sqrt{lipid} = 1.0)$$

$$\text{no. lysine residues per PI } (\eta) = \frac{\frac{\%L \times d_1}{\text{mol. wt. lecithin}}}{\frac{\%PI \times d_1}{\text{mol. wt. PI}}} = \frac{A_{lysine}}{A_{PI}}$$

$$\sigma = \frac{(\eta - 1)}{A_{PI}}$$

SUMMARY

1. Addition of charged lipid, CTAB to pig-liver lecithin causes swelling of the lamellar repeat distance but has no effect on the lecithin bilayer thickness. These results, being qualitatively the same as those of Gulik-Kryzwicki, confirm his experiments.

2. The lamellar repeat distance, d, for samples of egg lecithin centrifuged in glucose and sucrose solutions, goes through a maximum at 22 weight per cent of sugar solution. This is in qualitative agreement with theoretical predictions of V. A. Parsegian and his collegues.

3. Until very high sucrose concentrations, the thickness of the lecithin bilayer does not change with addition of sucrose solutions.

4. The decrease in d_1 at high sucrose concentrations appears to be associated with the crystallization of the sucrose.

5. The lamellar repeat distance for samples of egg lecithin centrifuged in dextran solutions decreases as the concentration of the solutions increase.

6. The thickness of the lecithin bilayer, in the presence of aqueous dextran solutions, appears to decrease significantly.

7. The assumption that dextran and lecithin form two separate aqueous phases adequately explains the results from the gravimetric and centrifuged samples.

8. Experiments showed that interactions between lecithin and; glucose, sucrose and dextran solutions, were reversible.

9. Attempts to test the dependence of the structural parameters of the monocaprin lamellar phase on the dielectric properties of the aqueous space, by the use of sucrose solutions, proved inconclusive.

10. Poly-l-lysine forms a lamellar complex with mixtures of the phospholipids, lecithin and phosphatidyl inositol, (PI), in which the interaction between lipid and protein is strictly ionic in nature.

11. As the lecithin content in the precipitates increases, the amount of PI bound and the charge density remains relatively constant, while the water content increases.

12. Single poly-l-lysine molecules appear to be binding to adjacent lipid bilayers prohibiting swelling of the structure.

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