STUDIES ON CELL ADHESION

by

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C O N T E N T S

SUMMARY

A single cell suspension was prepared from the neural retina tissues of seven days embryonic chicken. The cell suspension was allowed to settle on the clean glass surface by incubating for one hour at 38° C in a serum medium of Eagle's fluid. The microscopic observation showed that the majority of the cells were single and strongly adhered to the untreated glass surface. It was noticed that the interaction between glass and cells was strong enough to resist centrifugal forces.

Introduction of the lipid (distearoyl or dilinoleoyl phosphatidyl choline) onto the glass surface affected cell adhesion. Organisation of the oriented films on the glass were done on the water surface by the Blodgett and Langmuir techniques and for the unoriented films the lecithin was spread on the glass surface. When the lecithin monolayer was organised on the glass surface the cells became less adhesive to the glass. Adhesion decreased as more lipid was added to the glass surface. The cells kept their round form while being adhesive to the lecithin surface. The observations with C^{14} labelled lecithin glass surface showed that a large amount of C^{14} labelled phospholipid was taken into the cells when they were incubated in contact with the surface for one hour at 38 ° C in a serum medium and lysolecithin was released into the medium. But when the cell suspension was shaken to prevent contact with lecithin it was observed that much less lysolecithin was released and less labelled lecithin incorporated into the cells.

This suggests that phospholipases were involved in the production of lysolecithin so that contact is required for their action. It is known that the lysolecithin diminishes the adhesion of cells, therefore the observations obtained from this experimental work indicate that the neural retina cells will not adhere to a lecithin surface because of the released lysolecithin after lecithin incorporation into the cells during incubation period.

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CHAPTER 1

I N T R O D U C T I O N

Most cells can adhere to non-cellular substrates. Reactions of cells in culture to contact with their glass substratum have recently been treated by several investigators. Abercrombie (1958) and Weiss (1958) have considered two types of cell contact, namely that between one cell and another, which offers a basis for specific interactions and that between a cell and non-cellular objects, which probably expresses some of the simpler and more basic points of cell behaviour. Cell interactions with glass surfaces are generally considered in terms of a balance between electrostatic repulsion by cell surface charge and attractive forces of the London Van der Waal's type (Curtis 1962). Curtis (1967) in a review of the subject of cell adhesion has pointed to a considerable body of evidence for the existence of two classes of cell adhesion. The first is an adhesion with separation of less than 20 Å, the cell being in molecular contact and will not easily be dispersed. The second type of adhesion occurs with 100 to 200 Å separation between the membranes. Curtis (1966) has suggested that cell adhesion is the result of the balance between opposing physico-chemical forces.

The nature of the physico-chemical groups within cell peripheries affects their abilities to adhere to and to separate from each other. Studies on preparation of isolated lipids have provided strong evidence that phospholipids in aqueous systems are often organised bimolecular leaflets (Glauert and Lucy 1968). For some time it has

been generally assumed that the lipids of cell membranes are always arranged in this configuration. The various components of cell membranes, particularly their constituent protein materials, lipid, cholesterol polysaccharides, metal ions, water are all factors which introduce an enormous degree of complexity. The presence of lipids in cellular membranes was first proposed in the nineteenth century to account for the observed relationship between lipid solubility and the velocity of penetration of compounds into cells. Overton postulated in 1902 that the plasma membrane is composed of a thin layer of lipid. In 1926, Gorter and Grendel found that the lipid content of hemolyzed erythrocytes was sufficient to form a continuous layer 60 to 80 Å thick over the entire cell surface and postulated that the plasma membrane is composed of a double layer of lipid molecules. The most important phospholipids of cell membranes are diacylphosphatidylethanolamines, phosphatidyl choline (lecithin), phosphatidyl serine and phosphatidyinositol which can be considered as long molecules with two fatty acid chains usually showing distribution in chain length and also in their degree of saturation. The various types of lipids of the biological membranes can also be considered part of macromolecular array either in combination with carbonhydrate or with protein by forming the lipoprotein molecules of the basic structure of cell membranes whether mitochondrial, nuclear, endoplasmic reticulum, or plasma membrane. Two main functions can be attributed to the plasma membrane. The first is concerned with transport and the second with cell contact which provides a means of interaction or communication by transfer of chemical information

from one cell to another. Proteins in the cell membrane probably have many functions. Mitchison and Swann (1954) suggested that the membrane's elasticity and mechanical ability to expand and contract could be due to fibrous proteins. The lipid layer between two layers of proteins in the membrane are visualized as attaching to protein by interaction of polar functional groups and hydrocarbon chain of the lipid inlayers are visualized as interacting with each other, perhaps by interdigitation of chain. Lenard and Singer (1966) proposed a structure of the plasmalemma based on spectroscopic and optical ratatory dispersion studies. They suggested that the ionic and polar heads of lipid molecules, together with all of the ionic side chain of the structural proteins are on the exterior surface of the membrane in Van der Waal's contact with the bulk aqueous phase. Sequences of the structural proteins consisting predominantly of non-polar side chain are in the interior of the membrane, together with the hydrocarbon tails of the phospholipids and the relatively non-polar lipids such as cholesterol. In particular, the helical portions of the protein are interior, where they are stabilized by hydrophobic interactions. Electron microscopy has thrown some light on the fine structure of the plasma membrane. The definite thickness of plasma membranes of 60 to 100 Å have been observed at the surface of cells by electron microscopy. Under higher microscopic resolution the plasma membrane of most types of cells appeared three-layered. Each of the three layers is about 25 Å and the middle layer was found to be less dense than the other two (Zetterquist 1956). Robertson in 1959 called this structure unit membrane which is also

observed in most intracellular membranes. It was noticed that a small bridge crossed the light central layer and it was suggested that they were pores. Studies of the permeability of the plasma membrane showed that the maximum porosity of the membrane would be one pore per protein molecule so that inorganic ions would be able to pass between two protein molecules.

Electron microscopic observations showed that the two cells were in close contact and appeared as dense line separated by space of 110 to 150 Å and contained a material of low electron density in that space. This intercellular component was considered as a kind of cementing substance. Many authorities (Dervichian 1949) believed that the primary basis of lipoprotein structure is colombic binding between the ionic lipids such as lecithin. Curtis pointed out that the cell surface can be seen as a layer of low dielectric constant due to the presence of lipid bilayer and a variety of forces act to stabilize the bilayer structure.

The thermodynamic considerations and experimental results discussed fit in with the idea of a mosaic structure for the proteins and lipids of membranes by Singer and Lenard (1966). Singer and Nicolson suggested that the globular molecule of the integral proteins alternate with sections of phospholipid bilayer in the cross section of the membrane. The globular molecules are postulated to be amphipathic as are phospholipids and is structurally asymetric with one highly polar end and one non-polar end (Lenard, Singer and Wallach 1966). The highly polar region which is the ionic amino acid residues is bound covalently to the saccharide residues to form glycoproteins and is also in

contact with the aqueous phase on both sides of the intact membrane so that the integral protein molecule with the appropriate size and structure may transverse the entire membrane. The non-polar end was embedded in the hydrophobic interior of the membrane. It was suggested that the phospholipids of the mosaic structure are predominantly arranged as an interrupted bilayer, with their polar groups in contact with the aqueous phase and a small portion of the lipid more intimately associated with integral proteins.

Most lipoproteins are fairly loose associations and rather unstable and exchange their lipid moieties quite readily with lipid in the environment. The uniformity of lipoprotein combination in a particular cellular membrane suggests that the specificity of the binding of lipids to proteins may depend on a precise steric arrangement (Dawson I968). The organization of the lipoprotein microstructure will depend on multiple attachment and matching of polarity which will determine the combination of enzymes with their substrate, for example, phospholipase reactions. Lipoproteins often possess enzymic activity and soluble enzymes can be extracted from lipoproteins by various agents which disrupt the lipid protein association (Dawson I972). Many lipoproteins are attached to membranes where a whole range of lipids and proteins are arranged together and for this reason much of the work on lipid protein interactions has been done with model systems.

The model of the plasma membranes as a lipid bilayer coated with protein was first suggested by Danielli and Davson (I934-35). Model system experiments can explain certain lipid-protein interactions and they may tell us about what kind of membrane action

takes place in the cell. Lipid such as fatty acid, phospholipids, cholesterol and cholesterol ester, can be packed in single or bilayers. The orientation of the lipids within such structures is partly determined by the presence of the polar heads of the hydrocarbon chains. When polar lipid such as phosphatidyl choline (lecithin) is dispersed on a water it will tend to orient in a lamellar array. It is known that the positive and negative charges on the head group of phosphatidyl choline balance each other so that there will be no repulsion between them. The charged or strongly polar group associate with the water molecules while its non-polar tail (fatty acid site of hydrocarbon chain) associate with each other by Van der Waal's forces which are weak and operate short distances only. These properties of lipids have been studied by forming films on the water surface by Adam and Davies and Rideal (1963). Since the biological membranes are known to result from interaction between lipids and proteins the model and artificial monomolecular films are of considerable biological interest. The so-called film balance devised by Langmuir in 1917 is still the principal instrument for the study of the films on glass surface by dipping technique. Blodgett in 1935 observed that the amount of surface active material that could be deposited on a glass slide depended on several factors. He introduced the concept of X and Y type films. A definition of these films was given in terms of the radio between the area occupied by the monolayer on the solid substrate and the area occupied on the aqueous surface. An ideal Y-type film defined as multilayer system for both upward and downward dipping trips and X-type film is defined as a layer system

for the downward movement of the slide and zero for upward trips.

The present work is concerned with studies of contact interactions of the neural retina cells with clean glass coverslips and with lecithin covered glass surfaces.

The introduction of a lecithin layer onto glass and the incubation of cells onto an adsorbed layer of serum changes the nature of the cell/glass interaction. The observed reactions of these cells were changed in respect of the rate of attachment and spreading on a lecithin film compared with glass and the adhesiveness of the cells was diminished by lysolecithin which was released into the incubation medium by cells from the lecithin layers.

CHAPTER 2

MATERIALS AND METHODS

2.1 THE COVERSLIPS

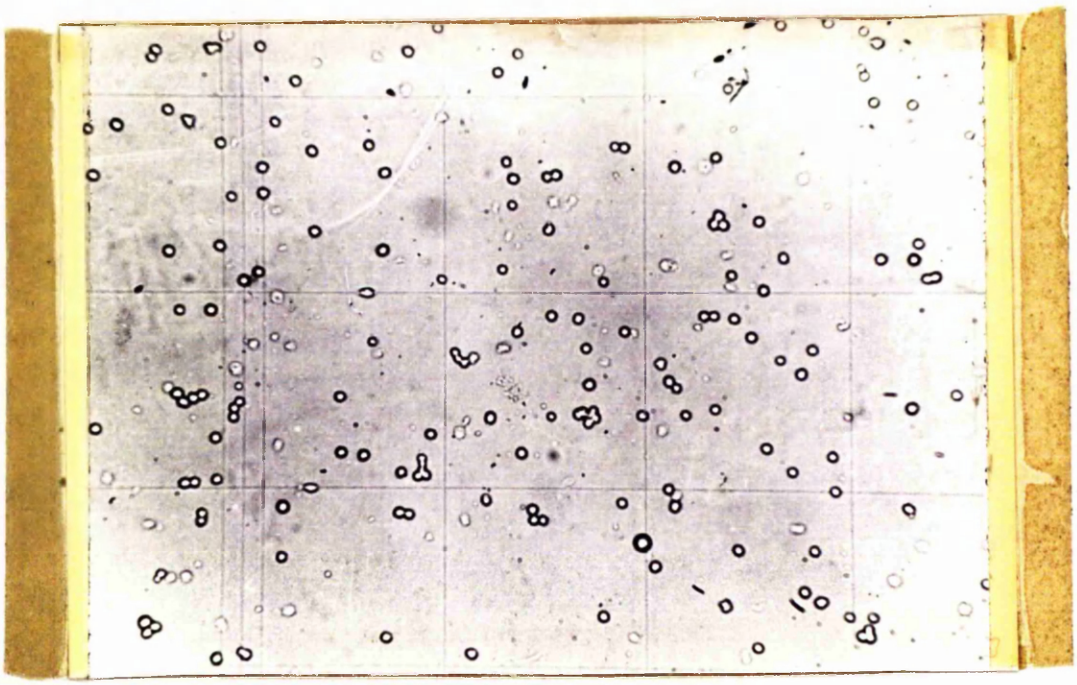
Glass coverslips 14.72cm^2 were cleaned in absolute alcohol/nitric acid. After this point two different cleaning methods were used as follows:

a) After rinsing with tap water they were rinsed with distilled water once, and they were then further washed by boiling three times in fresh changes of distilled water and dried in air.

b) After washing with nitric acid they were rinsed with tap water once, distilled water and ethanol. They were brought into warm KOH and allowed to remain in it for one second. They were rinsed again with tap water and transferred to the other dish which contained diluted nitric acid. Once more, rinsing was made under the tap water and distilled water. They were then separated and dried in air.

2.2 CELL SUSPENSION

The eyes were removed from seven days chicken embryos (De Kalb strain) and placed in Hanks solution. The neural retinae were dissected away from the pigmented retinae with forceps. The tissues were transferred to a centrifuge tube. They were washed twice in about 4ml CMF (Calcium and Magnesium free Hanks' medium pH 7.8). The tissues were incubated in about 3ml trypsin concentration [0.25% Difco trypsin in Tris Saline (NaCl 8g, KCl 2ml(1990), Na_2HPO_4 0.1g, Tris 3g, Phenol Red 1.5ml, Penicillin 1 x 10^5 units, Streptomycin 0.1g)] for 20 minutes at room temperature. The trypsin solution was removed without disturbing the tissue by pipetting and the tissues were washed with cold CMF twice. One drop



An initial single cell suspension also containing a few residual clusters.

Brightfield illumination
Magnification - x 248

of bovine serum was dropped with pasteur pipette to act as a trypsin inhibitor before adding about 5ml cold CMF into the tube. The tissues were gently pipetted until they were dispersed into single cells. To prepare a good single cell suspension the cells were centrifuged at 300rpm for one minute and the supernatant was transferred to the other clean centrifuge tube and centrifuged again at 1800rpm for 5 minutes at 4 °C. The supernatant was poured off. The cells at the bottom of the tube were pipetted gently after the addition of cold fresh CMF to resuspend them. The cell suspension was diluted by adding 2ml of this suspension to 10ml culture medium (MEM) to give about 0.25×10^6 cells per ml.

12ml of this cell suspension was poured into the petri dish which contained two clean glass slides. The petri dish was incubated at 38 °C for one hour in a dessicator which had a water saturated atmosphere containing 5% CO_2 in air. The cells were allowed to settle and attach to clean glass coverslips. After incubation the petri dish was taken out from the dessicator and coverslips were removed gently with forceps. The coverslips were transferred to 35ml cold MEM (Eagle's MEM, 10% Calf serum and 2% embryo extract) medium and were centrifuged for five minutes at 300 rpm. The adhesiveness of the cells was tested by centrifuging the coverslips in a tube. After centrifugation the coverslips were removed from the tubes (Ambrose and Easty technique 1960). The coverslips were inverted and mounted on a slide. The edges of the coverslips were sealed with vaseline in order to prevent the cells on the coverslips drying. The adherent cells in an area were counted under the phase microscope with a 10X objective. The percentage of cells sticking to the coverslips is calculated from the equation:

Percentage of cells sticking

$$= \frac{\text{No. of cells sticking} \times 100}{T.\frac{a}{A}}$$

Where

T = the total number of cells in petri dish

A = the area of petri dish (cm^2)

a = the counting area under microscope (cm^2)

2.3 CELL ADHESION TO ORIENTED MONO AND MULTILAYER FILMS

Coverslips were coated with lecithin layers by the Blodgett technique.(1935)

A clean beaker of 12.5cm diameter was filled with approximately 2000ml of distilled water. The water surface was allowed to become smooth. Unlabelled lecithin (distearoyl calbiochem) 5mg/ml in $CHCl_3$ /MEOH solution was carefully dropped onto the surface from a 10μl syringe carefully positioned over the smooth water surface. The lecithin was added drop by drop in such a way that each drop was watched until a given drop suddenly formed a packed film on the surface and did not penetrate into the water. When approximately 4μl lecithin had been added the drops did not spread as quickly as previously, and gave what appeared to be a condensed layer and remained white for a few seconds at the dropping point which indicated that sufficient lecithin had been added. Two coverslips were then carefully floated on the surface and allowed to remain there for a few seconds before being gently removed and dried in air on a petri dish. Hence a thin film of either a monolayer or a few layers should be deposited on the surface of the slides. Experiments with C^{14} labelled lecithin described later demonstrates that many layers were formed. After removing the floating slides gently with forceps another aliquot of

lecithin solution in the syringe was added to the water surface as before until the surface was covered sufficiently with lecithin. One of two dried slides was allowed to float again on the water surface. This was repeated another four times under the same conditions as before. Therefore, by adding lecithin each time, a multilayer film six times the original thickness should be deposited on the glass surface. Approximately 4μl of lecithin solution containing 20μgm lecithin was used in the preparation of the film on the glass of area 14.72cm^2 on the dish and hence 2.4μgm adheres to each coverslip giving a theoretical thickness for each layer of 14.7Å . The two slides which had monolayer and multilayer film on their surfaces were transferred into the petri dish. 2ml of cell suspension which contained about .25x10^6 cells per ml in cold CMF was resuspended into 10ml of culture medium (MEM) in the conical tube. This suspension was poured into the petri dish and the cells were allowed to settle on the coverslips which had the lecithin films on their face. The same amount of cell suspension was prepared and added to another petri dish which also had two clean coverslips without mono or multilayer film on their surfaces as a control. The two petri dishes were placed in a hot room and incubated for one hour at 38 ° C. After incubation the coverslips were transferred with approximately 35ml of fresh medium (MEM), into 50ml centrifuge tubes and spun at 300rpm for 5 minutes. The adherent cells stayed on the coverslip and non-adherent cells were transferred to the medium. The adhesive cells on the coverslips were counted under 10X objective with 10X additional magnification in a known 50 square area.

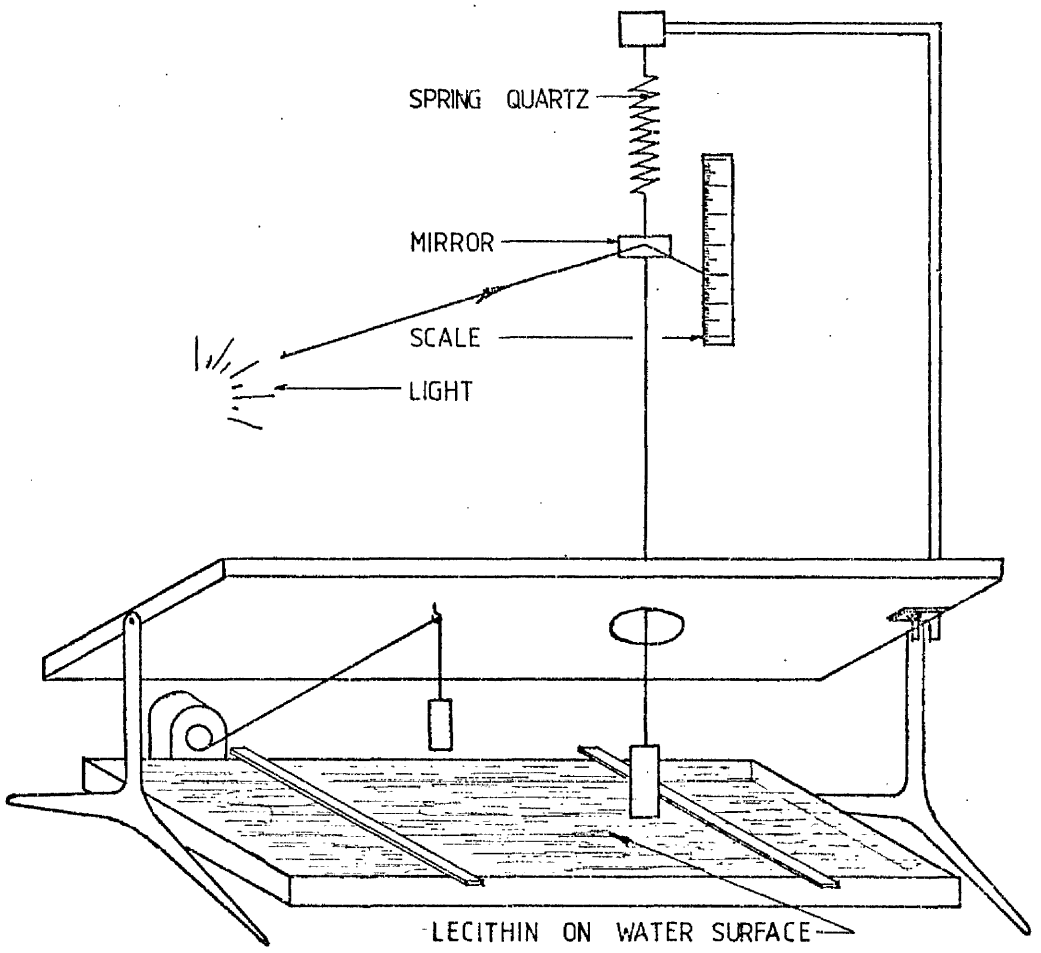
2.4 LANGMUIR TROUGH MEASUREMENT OF FILM THICKNESS (Langmuir 1917)

The coating was done by spreading a film on a distilled water surface by dissolving the test material which was distearoyl lecithin and dilinoleoyl lecithin in the solvent chloroform-methanol and allowing the monolayer to become firmly adsorbed onto a clean glass surface. Glass coverslip surfaces were cleaned before use in order to avoid contamination.

MEASUREMENT OF FILM THICKNESS:

The clean glass shallow trough was heated in the oven for 30 minutes. After heating, the shallow trough was waxed using a clean tissue to obtain a smooth wax coat on the trough and the three glass barriers were also waxed. The wax coated glass trough was taken under the carrier which had a galvonometer scale on the top. The trough was filled with distilled water on which the lecithin solution was spread. The water surface was swept by moving the wax coated bar across the trough in order to check the purity of the freshly distilled water surface. The thick clean slide was placed onto the hook which was connected to galvonometer scale by passing through the hole under the carrier. Therefore, the surface pressure exerted by the film was measured by the sensitive floating and suspended balance. Before the thick slide was placed in position it was cleaned with alcohol, diluted nitric acid and ethanol. The second wax coated bar was moved towards the suspended balance so that the distilled water surface was checked for purity by surface tension measurement again before the lecithin was added on to the surface. The two bars were positioned 19 cm apart by leaving the suspended balance between them. The balance was calibrated for given pressures with weights.

10 μl cold distearoyl lecithin solution containing 50 μ gm lecithin in



LANGMUIR TROUGH MEASUREMENT OF FILM THICKNESS.

$CHCl_3$ /MEOH was allowed to spread over the water surface between the two barriers without penetrating the water. After adding the lecithin the galvonometer scale reading was recorded in order to calculate the surface pressure. The clean thin coverslip was dipped into the trough very slowly by using a slow drive electric motor. The liquid film on the water surface was brought into contact with solid glass surfaces when the coverslip broke through the water surface. The coverslip can be regarded as a Wilhelmy plate. The slide was lifted up slowly after it touched the bottom of the tank. Dipping the slide into the trough was repeated another five times and it was dried in the air. The same method of coating the coverslips measured for dilinoleoyl lecithin as well. At the end of the experiment the trough was emptied in order to prepare another film on the other clean glass coverslip but this time the preparation was made at a different surface pressure by using the same technique and the same amount of lecithin. Some preparations were made with dilinoleoyl lecithin as well. The film on the glass coverslips were obtained under different surface pressures with last preparation.

THE CELL SUSPENSION:

The cell suspension was prepared as described before and it was diluted in 12ml culture medium (MEM and 5% calf serum) to give 0.25 x 10^6 cells per ml. The film coated coverslips were transferred into the petri dish and 12ml cell suspension was poured into each petri dish. The petri dishes were incubated for an hour at 38 ° C. After incubation the coverslips were centrifuged at 300rpm for 5 minutes. The coverslips were taken out from the centrifuge tubes and they were placed under the microscopic field to examine the adherent cells on the film contained coverslip surfaces. The two clean coverslips without film on their surfaces were also incubated with 12ml cell suspension as a control.

2.5 MEASUREMENT OF PHOSPHOLIPID ADHERENT TO COVERSLIP

a) Before Exposure to Cells

10 μl C^{14} labelled lecithin (in New Zealand Nuclear NEC-588 phosphatidyl choline - C^{14} (U) from algae grown in carbon C^{14} dioxide) was used during the experiment. 10 μl C^{14} labelled lecithin solution specific activity 4×10^6 dpm/μgm in benzane was put into the tube and dried with nitrogen gas. 25 μl cold lecithin solution containing 125 μgm (5mg/ml) lecithin in $CHCl_3$ /MEOH was added to the tube with 75 μl cold $CHCl_3$ /MEOH. A 5 μl sample was taken to count the radioactivity in the mixed lecithin. A clean beaker of 12.5cm diameter was filled with approximately 2000ml of distilled water. When the water surface became smooth the 2 μl labelled lecithin and cold lecithin mixture was positioned on the water surface. The two clean coverslips were allowed to come into contact with the water surface. The slides were removed from the dish and dried in the air. Other coverslips were covered with thicker layers repeating this technique but using 4 μl or 8 μl or 16 μl aliquots of lecithin solution. 3.3cm^2 area was cut off from every glass slide to measure their radioactivity. Activity was measured in a scintillation counter by dissolving the liquid from the coverslips with the scintillant 2.5% PPO in toluene. The cell suspension was prepared as described previously. The film contained glass coverslips were placed into the petri dish. 12ml cell suspension which contained 0.25×10^6 cells per ml was poured into the petri dish. The same amount of cell suspension was added to another petri dish which also had two clean coverslips as a control. The petri dishes were incubated for one hour at 38° C.

b) After Exposure to Cells

Cells were prepared and cultured on the coverslips as described above. After incubation the slides were removed and transferred into the fresh medium (MEM 5% calf serum) contained in a conical tube for centrifugation. After centrifugation the slides were placed under the microscopic field to count the adherent cells to the glass surfaces. The calculation of the percentage of adherent cells was made by the method described earlier.

2.6 CELL ADHESION TO UNORIENTED FILMS

Comparable experiments were carried out using unoriented films. A mixed labelled lecithin preparation containing either cold dilinoleoyl or distearoyl lecithin and labelled mixed lecithin was prepared as described below. These films were much thicker than the oriented films which provide the opportunity of examining chemical changes in the liquid after incubation by TLC.

a) 10μl C^{14} labelled lecithin solution (NEC-588 phospatidyl choline - C^{14} (U) from algae grown in carbon C^{14} dioxide) specific activity 4×10^{6} dpm/μgm in benzene was added to 10μl cold distearoyl or dilinoleoyl lecithin solution containing 50μgm (5mg/ml) lecithin in $CHCl_3$ /MEOH. 50μl $CHCl_3$ /MEOH was added on this lecithin mixture as a matter of preparing films on the coverslips. To measure the specific activity of this mixture a 5μl sample from this was dried under nitrogen gas in a scintillation vial before it was counted in the scintillation counter. 10μl lecithin mixture was dropped onto a coverslip. The drop on the coverslip was spread with the other coverslip by making an angle of

approximately 45° between them and ensuring that radioactive lecithin remained on the coverslip. Spreading was continued until the drop disappeared on the slide surface so that unoriented film was produced on the coverslip. The coverslip was allowed to dry in the air. After evaporating the solvent, an area of 3cm^2 was cut from the two coverslips with a diamond point in order to count the radioactivity on the known slide area to measure average film thickness before the slides incubated with cell suspension.

Measurement of Cell Adhesion:

The same amount of cell suspension was prepared and cultured on the coverslips as described earlier. After incubation the coverslips were cut into strips of 3cm^2 with a diamond point. The strips were used to measure lecithin on the coverslips and incorporation into the cells which had settled on the glass surface during one hour incubation at 38 °C. After calculation of the percentage of adherent cells to the glass, incubation medium and cells were collected for measurement of radioactivity.

b) Measurement of Radioactivity Released on Culture:

The distribution of radioactivity between coverslip, medium, adherent cells and non-adherent cells after incubation was followed by the techniques described for the comparable experiments with oriented films. See particularly Figure 2.I for flow diagram.

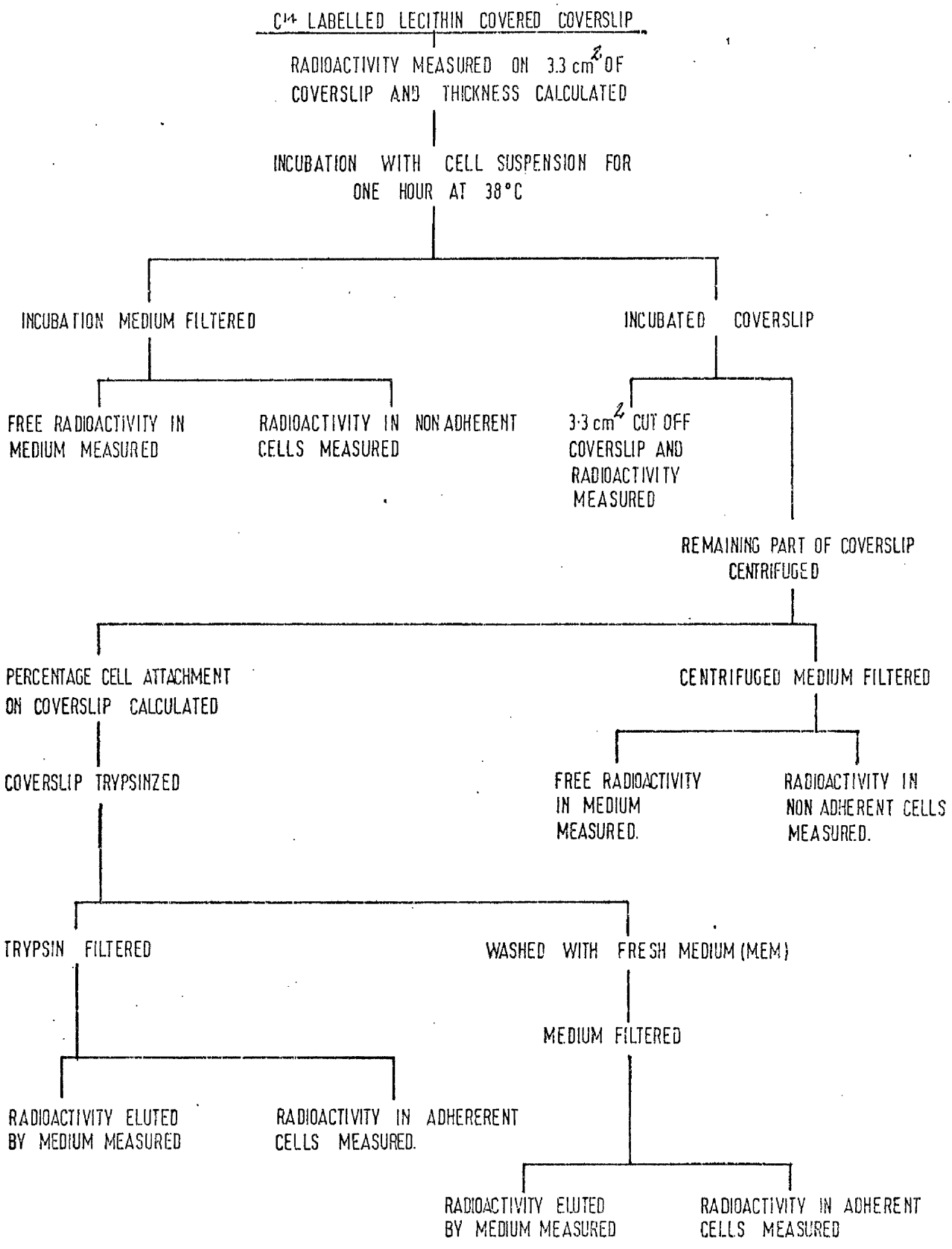


Fig. 2.I Flow Diagram for Cell Adhesion to Unoriented Films.

2.7 CELLS IN CONTACT AND NON-CONTACT WITH LECITHIN SURFACE

The two types of experiment were carried out. During the first experiment the cells were allowed to come into contact with lecithin covered glass surface. The preparation of this experiment was exactly the same as the last experiment described on the previous page, except the amount of radioactive lecithin and cold lecithin were increased. For the second experiment the cell suspension was placed in C^{14} labelled lecithin covered conical flask by using the shaker bath in order to prevent cell contact with glass surface during incubation. The purpose of these experiments was to discover if cells in contact released lysolecithin from a lecithin surface.

A - Cell Adhesion on the Film Covered Surface:

a) Before Exposure to Cells

In this experiment 25μl C^{14} labelled lecithin was mixed with 50μl cold lecithin (5mg/ml in $CHCl_3$ /MEOH solution) and 100μl $CHCl_3$ /MEOH 50:50 added to the mixture. A 5μl sample was taken from the mixture and its radioactivity measured on the scintillation counter. 10μl mixture lecithin was spread on the thin coverslip with another slide as described previously. After the coverslip dried 3cm^2 area was cut off from the slide to measure the gain in radioactivity on the glass surface.

b) After Exposure to Cells

12ml cell suspension was prepared for each petri dish and the coverslips were incubated all as before. After incubation a 3cm^2 area was cut off from the slides again to measure the remaining radioactivity on the slide after incubation. The other parts of the coverslips were centrifuged in about 35ml fresh MEM medium for 5 minutes at 300rpm. After centrifuging, the coverslips were placed under the microscopic field to examine the

adherent cells on the film coated glass surfaces. It was found that there were no cells on the surfaces. Medium and cells were collected for measurement of radioactivity. The incubation medium and centrifuged medium were filtered through the 0.2 μm pore size milipore filter to collect the floating cells in the mediums.

c) Extractions of Liquids for TLC

The incubation medium which passed through the millipore filter was extracted with 5ml $CHCl_3$ /MEOH 50:50 in a universal bottle and allowed to stand until separated at room temperature. After settling, the extract was collected into the clean universal bottle by pipette. The extraction from the incubation medium was repeated four times using 5ml pure $CHCl_3$ each time by saving the extracts into the universal bottle each time and it was completed by 5ml $CHCl_3$ /MEOH. The collected extract was dried under nitrogen gas. The medium from the centrifugation was filtered to obtain unadherent cells from the culture and to isolate any substance released by the cells for identification by chromotography.

d) TLC Methods

The 20 x 20cm glass plates (Nachey-Nagel) were used. 50 μgm standards of lecithin were introduced with 10 μl syringe about 2.5cm from the lower edge of the plate. An extracted sample from the incubation medium and an extracted sample from the centrifuged medium was re-extracted with 50 μl $CHCl_3$/MEOH.

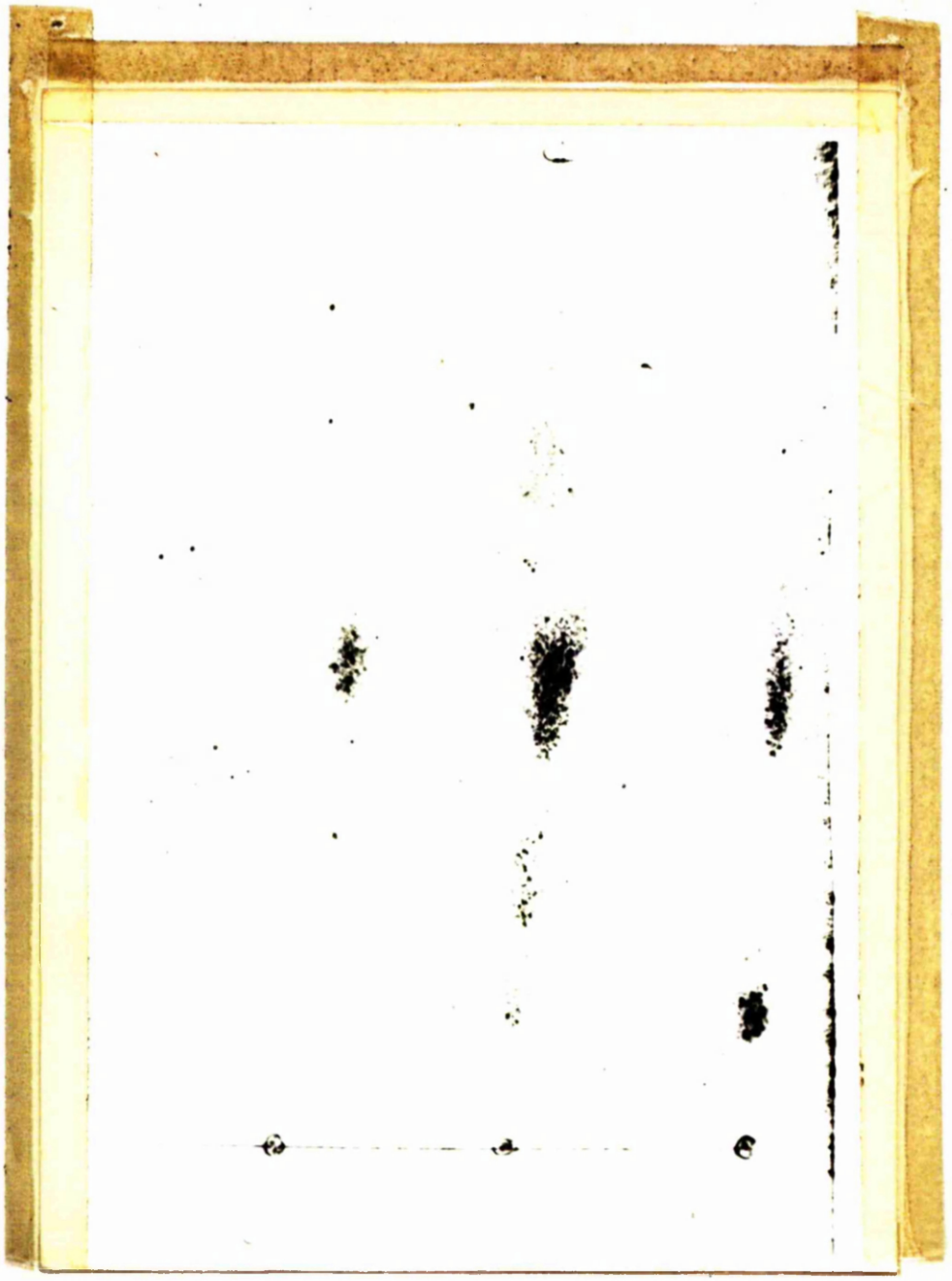
A 25 μl re-extract from incubation medium and 50 μl re-extract from centrifuged medium were spotted on the starting line separately. The chromotography tank was filled to a depth of approximately 2cm with solvent I (Chloroform 100ml , Methanol

60ml , Acetic acid 16ml , Distilled water 8ml) which run in the tank the ascending mode. Before the plate was placed in the tank the atmosphere of the tank was saturated with the solvent vapor. The movement of the liquid phase was stopped when the front had reached a sufficient distance which was 10cm from the starting point. The plate was removed from the tank and dried. The position of the lipids were revealed by spraying Malachite green or with Mb-blue. Malachite green reveals lysophospholipids as white spots. Identities of spots were determined by comparison with the standards. The compounds were reccvered by scraping the adsorbent from the plate where the spray indicated a zone to be present and they were transferred into the vials to measure radioactivity.

B - The cells not in contact with a lecithin surface; release of lysolecithin

For this experiment the cell suspension was placed in C^{14} lecithin covered conical flask.

After 5μl C^{14} labelled lecithin was dried by oxygen-free nitrogen 20μl cold distearoyl lecithin (5mg/ml in $CHCl_3$ /MEOH solution) with 05ml /$CHCl_3$ /MEOH 50:50 was mixed with it. The radioactivity of the 5μl solution was counted. A clean conical flask (50 ml) was used during this experiment. The inside of the flask, especially the bottom, was covered with lecithin solution by pouring the labelled lecithin in the flask and shaking until the solution evaporated. 12μl cell suspension was prepared which was described as before and poured into the C^{14} coated flask and shaken in the shaker bath for an hour at 38 $^{\circ}$ C. The flask was removed from the bath and the medium was filtered through an 0.2μm pore size millipore filter to collect C^{14}



Extracted Centrifuged Medium	Extracted Incubation Medium	Standard

R_f value for Lecithin = 0.6
R_f value for Lysolecithin = 0.22

CHROMATOGRAM OF EXTRACTED INCUBATION AND EXTRACTED CENTRIFUGED MEDIUM FOR CELLS IN CONTACT WITH LECITHIN SURFACE.

incorporated floating cells in the medium. The filter was transferred into the vial to measure the radioactivity at the centillation counter. The rest of the medium was

TLC

TLC was carried out as described above. After extraction the incubation medium was dried under nitrogen gas. Dry extracted incubation medium was re-extracted with 50μl $CHCl_3$ /MEOH. 10μl of the standard lysolecithin and 25μl re-extracted incubation medium were placed on the glass plate 3cm from each other by microsyringe at the starting line.

CHAPTER 3

R E S U L T S

3.1 CELL ADHESION ON CLEAN GLASS SURFACE

The neural retina cells in a serum medium of Eagle's fluid rapidly attached when they were settled onto a clean glass surface for one hour at 38 °C. As shown in Table 3.1 an average of 65% of the cells became attached to the glass surface. Microscopic observation showed that the majority of the cells were individual and adhered on contact with the substratum.

Table 3.1 - Cell adhesion on clean glass surface

T x 10^6	Adherent Cells	
	No	%
2.26	86	76.6
"	73	65.0
"	88	78.4
"	112	99.8
3.39	107	63.5
"	110	65.3
2.88	92	64.4
"	83	58.1
3.0	72	45.4
"	65	43.7
"	90	60.5
"	90	66.5
"	99	66.5
"	91	61.1
"	89	59.8
	Average	65.0
	SD	13.3

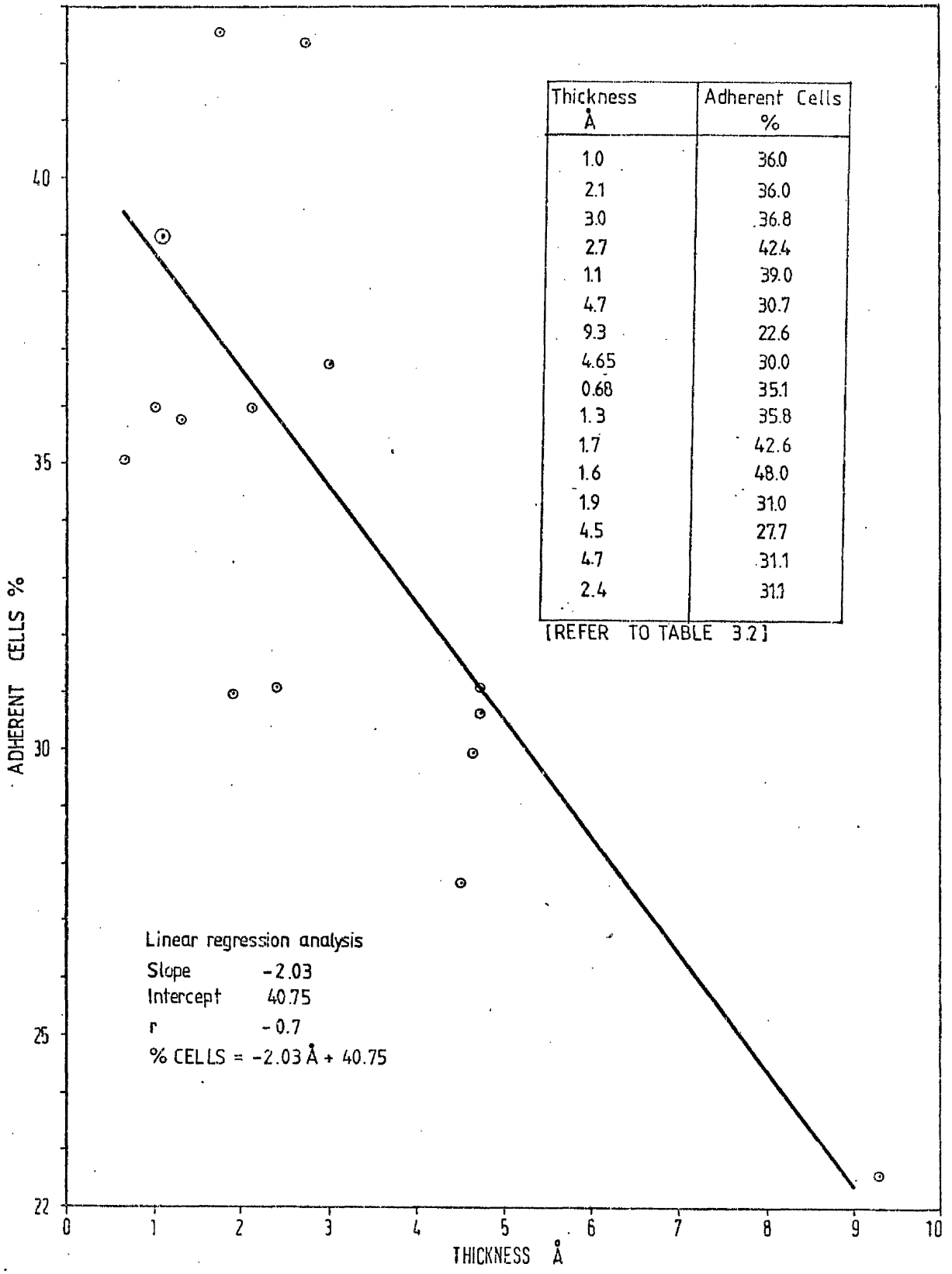
Area of petri dish (A) = 56.44 cm^2
Counting area of coverslips (a) = 0.0028 cm^2

3.2 CELL ADHESION ON C^{14} LABELLED DILINOLEOYL LECITHIN COATED GLASS SURFACE (BLODGETT TECHNIQUE)

The addition of the phosphatidyl choline (lecithin) on the glass surface caused a significant effect on adhesiveness of these cells in a serum medium. Attachment of the cells to the multi-dilinoleoyl phosphophatidyl choline layer in a serum medium was affected by the thickness of the lecithin layer such that as the thickness increased the percentage of cells sticking decreased as shown by the graph in Figure 3.1.

Table 3.2 - Cell adhesion on C^{14} labelled dilinoleoyl lecithin coated glass surface

No.	Dilinoleoyl Lecithin µl	cpm	Thickness Å	Adherent Cells No.	Adherent Cells %
A	T=2.52 x 10^6	-	-	-	-
	Background	30	-	-	-
	Control sample 5µl	7651	-	-	-
A1	2	37	1.0	-	-
A2	4	83	2.1	-	-
A3	8	116	3.0	-	-
A4	16	104	2.7	-	-
	After incubation				
A1	2	28	0.7	45	36.0
A2	4	16	0.4	45	36.0
A3	8	16	0.4	46	36.8
A4	16	39	1.0	53	42.4
B	T=2.76 x 10^6	-	-	-	-
	Background	22	-	-	-
	Control sample 5µl	5589	-	-	-
B1	2	32	1.1	-	-
B2	4	132	4.7	-	-
B3	8	267	9.3	-	-
B4	16	134	4.65	-	-
	After incubation				
B1	2	16	0.6	54	39.0
B2	4	32	1.1	42	30.7
B3	8	58	2.0	31	22.6
B4	16	77	2.7	41	30.0



Thickness Å	Adherent Cells %
1.0	36.0
2.1	36.0
3.0	36.8
2.7	42.4
1.1	39.0
4.7	30.7
9.3	22.6
4.65	30.0
0.68	35.1
1.3	35.8
1.7	42.6
1.6	48.0
1.9	31.0
4.5	27.7
4.7	31.1
2.4	31.1

[REFER TO TABLE 3.2]

FIG 3.I Variation of percentage of cells sticking with thickness for C^{14} labelled DILINOLEOYL LECITHIN coated glass surface. (Blodgett Technique). Results table 3.2

Table 3.2 (continued)

C	T=3 x 10^6	-	-	-	-
	Background	40	-	-	-
	Control sample 5µl	40493	-	-	-
C1	2	139	0.68	-	-
C2	4	274	1.3	-	-
C3	8	352	1.7	-	-
C4	16	325	1.6	-	-
	After incubation				
C1	2	62	0.3	52	35.1
C2	4	60	0.3	53	35.8
C3	8	79	0.38	63	42.6
C4	16	164	0.8	71	48.0
D	T=3 x 10^6	-	-	-	-
	Background	40	-	-	-
	Control sample 5µl	22693	-	-	-
D1	2	106	1.9	-	-
D2	4	522	4.5	-	-
D3	8	546	4.7	-	-
D4	16	276	2.4	-	-
	After incubation				
D1	2	32	0.3	46	31.0
D2	4	85	0.7	41	27.7
D3	8	160	1.4	46	31.1
D4	16	196	1.7	46	31.1

Area of petri dish (A) 56.44 cm^2
Counting area of coverslips (a) 0.0028 cm^2
Area of coverslip (C) 3.3 cm^2
Total number of cells in dish (T)
Settling time one hour at 38 °C

3.3 CELL ADHESION ON C^{14} LABELLED DISTEAROYL LECITHIN COATED GLASS SURFACE (BLODGETT TECHNIQUE)

The results of this experiment, as given in Table 3.3, do not show any relationship between thickness and cell adhesion although it is seen that a higher percentage of cells become detached compared with the clean glass surface (Table 3.1).

The fact that there is no relationship between the thickness and percentage of cells sticking may be due to the condensation of the lipid molecules after expended on the water surface and the organisation of the lipid molecules in varying thickness on the glass surface.

Table 3.3 - Cell adhesion on C^{14} labelled distearoyl lecithin coated glass surface

No.	Distearoyl Lecithin µl	cpm	Thickness Å	Adherent Cells No.	Adherent Cells %
A	T=3 x 10^6	-	-	-	-
	Background	20	-	-	-
	Control sample 5µl	4551	-	-	-
A1	2	51	2.2	-	-
A2	4	26	I.2	-	-
A3	8	213	9.3	-	-
A4	16	126	5.5	-	-
	After incubation				
A1	2	25	1.0	48	32.3
A2	4	20	0.9	50	33.8
A3	8	127	5.5	48	32.3
A4	16	69	2.9	52	34.9
B	T=2.76 x 10^6	-	-	-	-
	Background	20	-	-	-
	Control sample 5µl	6996	-	-	-
B1	2	75	2.2	-	-
B2	4	180	5.4	-	-
B3	8	160	4.8	-	-
B4	16	281	8.4	-	-
	After incubation				
B1	2	32	0.96	44	32.0
B2	4	76	2.3	42	30.7
B3	8	96	2.9	49	35.8
B4	16	102	3.0	40	29.2
C	T 3 x 10^6	-	-	-	-
	Background	40	-	-	-
	Control sample 5µl	14360	-	-	-
C1	2	108	1.5	-	-
C2	4	337	3.1	-	-
C3	8	362	5.0	-	-
C4	16	417	5.7	-	-
	After incubation				
C1	2	8	0.1	4	29.8
C2	4	51	0.7	37	25.0
C3	8	141	1.9	47	31.8
C4	16	297	4.1	41	27.7

Table 3.3 (continued)

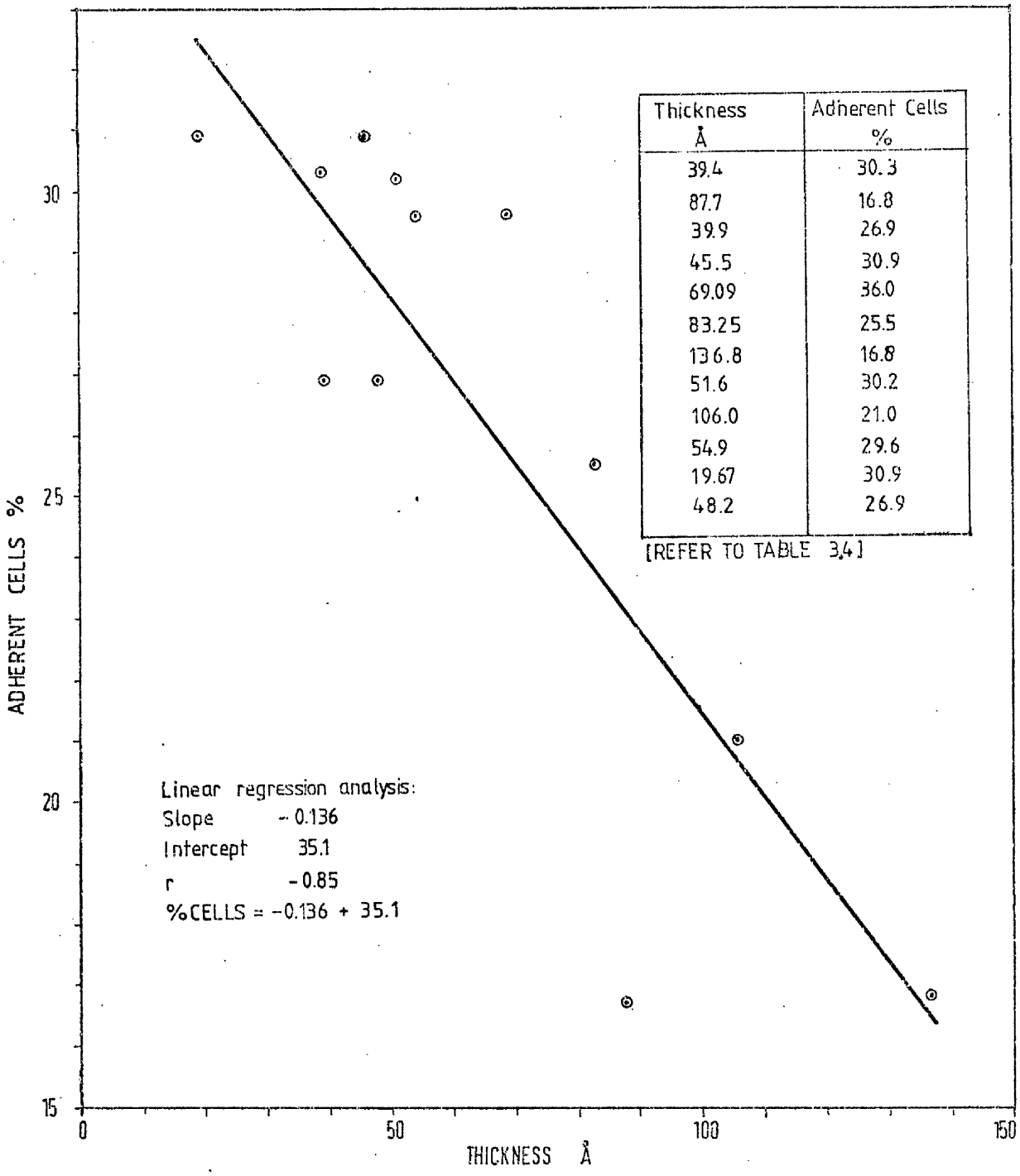
D	$T=3 \times 10^6$	-	-	-	-
	Background	20	-	-	-
	Control sample 5µl	23206	-	-	-
D1	2	295	3.4	-	-
D2	4	512	4.4	-	-
D3	8	539	4.6	-	-
D4	16	624	5.3	-	-
	After incubation				
D1	2	194	1.7	44	29.7
D2	4	239	2.0	37	25.0
D3	8	239	2.0	47	31.8
D4	16	217	1.84	37	25.0

Area of petri dish (A) = 56.44 cm^2
Counting area of coverslips (a) = 0.0028 cm^2
Area of coverslips (C) = 3.3 cm^2
Total number of cells in dish (T)
Settling time one hour at 38°C

3.4 CELL ADHESION ON C^{14} LABELLED DILINOLEOYL LECITHIN COATED GLASS SURFACE (UNORIENTED)

The results of the lecithin on cell adhesion as unoriented adsorbed film to glass are given in Table 3.4 and the relationship between thickness and percentage of cells sticking is shown by the graph in Figure 3.2. It is seen that a greater percentage of cells became detached as the thickness of the lecithin layer, which had either saturated or unsaturated non-polar chain, increased. Microscopic examinations showed that the neural retina cells remained in round form while being adhesive to the lecithin surface in serum medium and the majority of the cells were individual as in the previous experiment.

A large amount of lecithin incorporation occurred in the cells during incubation for one hour at 38°C on the lecithin surfaces. The results of the incorporated lipid into the neural retina cells and the mediums are given in Table 3.5 and Figure 3.3.



Thickness Å	Adherent Cells %
39.4	30.3
87.7	16.8
39.9	26.9
45.5	30.9
69.09	36.0
83.25	25.5
136.8	16.8
51.6	30.2
106.0	21.0
54.9	29.6
19.67	30.9
48.2	26.9

FIG 3.2 Variation of percentage of cells sticking with thickness for C^{14} labelled DILINOLEOYL LECITHIN coated glass surface. (Unoriented Film). Results table 3.4.

After incubation the floating neural retina cells, which did not attach to the dilinoleoyl lecithin layer, were collected from the incubation medium and approximately 31% of the radiation was measured on them. 46% of the radioactivity was found on the extracted incubation medium. The experiment was completed by collecting the adherent cells from the film surface in order to measure the incorporated radioactivity.

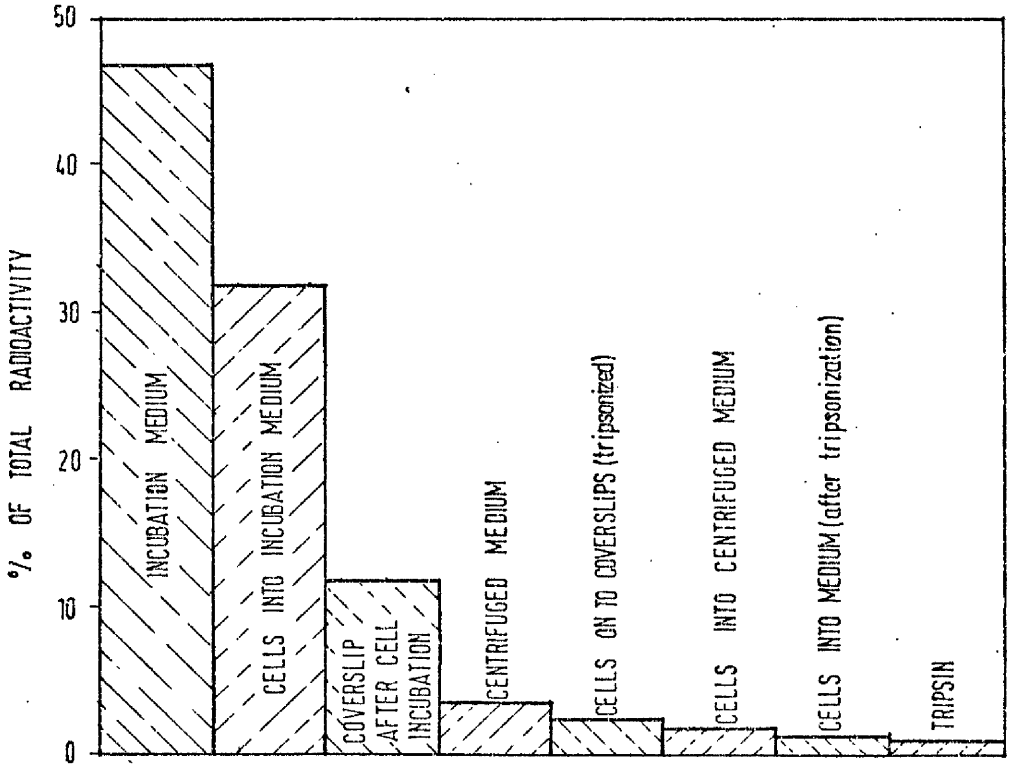


Fig 3.3 Distribution of radioactivity for C labelled DILINOLEOYL LECITHIN coated glass surface (Unoriented Film).

Results table 3.5

Table 3.4 - Cell adhesion on C^{14} labelled dilinoleoyl lecithin coated glass surface (Unoriented)

No.	Dilinoleoyl Lecithin	Radioactivity cpm	Thickness Å	Adherent Cells No.	Adherent Cells %
A	Background	27	-	-	-
	Control sample 5µl	18728	-	-	-
A1	Coverslip	3740	39.4	-	-
A2	Coverslip	8331	87.7	-	-
	After incubation				
A1	Coverslip	251	2.64	45	30.3
A2	Coverslip	264	2.78	25	16.8
B	Background	20	-	-	-
	Control sample 5µl	22509	-	-	-
B1	Coverslip	4564	39.9	-	-
B2	Coverslip	5207	45.5	-	-
	After incubation				
B1	Coverslip	278	2.43	40	26.9
B2	Coverslip	351	3.06	46	30.9
C	Background	27	-	-	-
	Control sample 5µl	15480	-	-	-
C1	Coverslip	5435	69.09	-	-
C2	Coverslip	6549	83.25	-	-
	After incubation				
C1	Coverslip	266	3.38	44	36.0
C2	Coverslip	251	3.19	38	23.5
D	Background	20	-	-	-
	Control sample 5µl	21387	-	-	-
D1	Coverslip	14868	136.8	-	-
D2	Coverslip	5608	51.6	-	-
	After incubation				
D1	Coverslip	842	7.75	25	16.8
D2	Coverslip	1241	11.42	45	30.2
E	Background	25	-	-	-
	Control sample 5µl	23807	-	-	-
E1	Coverslip	12851	106.0	-	-
E2	Coverslip	6645	54.9	-	-
	After incubation				
E1	Coverslip	366	3.025	31	21.0
E2	Coverslip	292	2.4	44	29.6
F	Background	20	-	-	-
	Control sample 5µl	38421	-	-	-
F1	Coverslip	3840	19.67	-	-
F2	Coverslip	9413	48.2	-	-
	After incubation				
F1	Coverslip	692	3.54	46	30.9
F2	Coverslip	396	2.03	40	26.9

Area of petri dish (A) = 56.44 cm^2
Counting area of coverslips (a) = 0.0028 cm^2
Area of coverslip (C) = 3.3 cm^2
Total number of cells in dish (T)
Settling time one hour at 38°C

Table 3.5 - Distribution of radioactivity for C^{14} labelled dilinoleoyl lecithin coated glass surface

(Unoriented Film)

	Experiment Reference (Table 3.4)										Average %
	B		C		D		E		F		
	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	
Coverslip after cell incubation	629	11.7	517	8.12	2083	16.9	658	8.02	1089	14.49	11.85
Cell into incubation medium on filter paper	2003	37.25	1293	20.32	4762	38.63	2730	33.29	2241	29.81	31.86
Cell into centrifuged medium on filter paper	100	1.86	36	0.57	469	3.8	168	2.05	79	1.05	1.87
Cell into coverslips (trypsinized)	332	6.17	45	0.71	303	2.46	38	0.46	75	1.0	2.16
Cell into medium (after trypsinization)	25	0.47	140	2.2	185	1.5	135	1.65	8	0.11	1.18
MEDIUM EXTRACTED											
Incubation medium	2095	38.96	41.48	65.18	3667	29.75	4159	50.71	3724	49.53	46.82
Centrifuged medium	132	2.45	185	2.91	718	5.83	279	3.4	216	2.87	3.49
Trypsin	61	1.13	30	0.47	140	1.14	34	0.42	86	1.44	0.9
TOTAL	5377	100	6394	100	12327	100	8201	100	7518	100	

3.5 CELL ADHESION ON C^{14} LABELLED DISTEAROYL LECITHIN COATED GLASS SURFACE (UNORIENTED)

The percentage of the adhesive cells to the distearoyl lecithin film surface decreased as the thickness of the film increased as shown in the graph in Figure 3.5, which is plotted from the results given in Table 3.6. Lecithin incorporation occurred in the cells during incubation as shown in the results obtained given in Table 3.7 and Figure 3.4 which shows that a large amount of radioactivity was measured in the cell after incubation. The cell remained in round form while being adhesive to the lecithin layer.

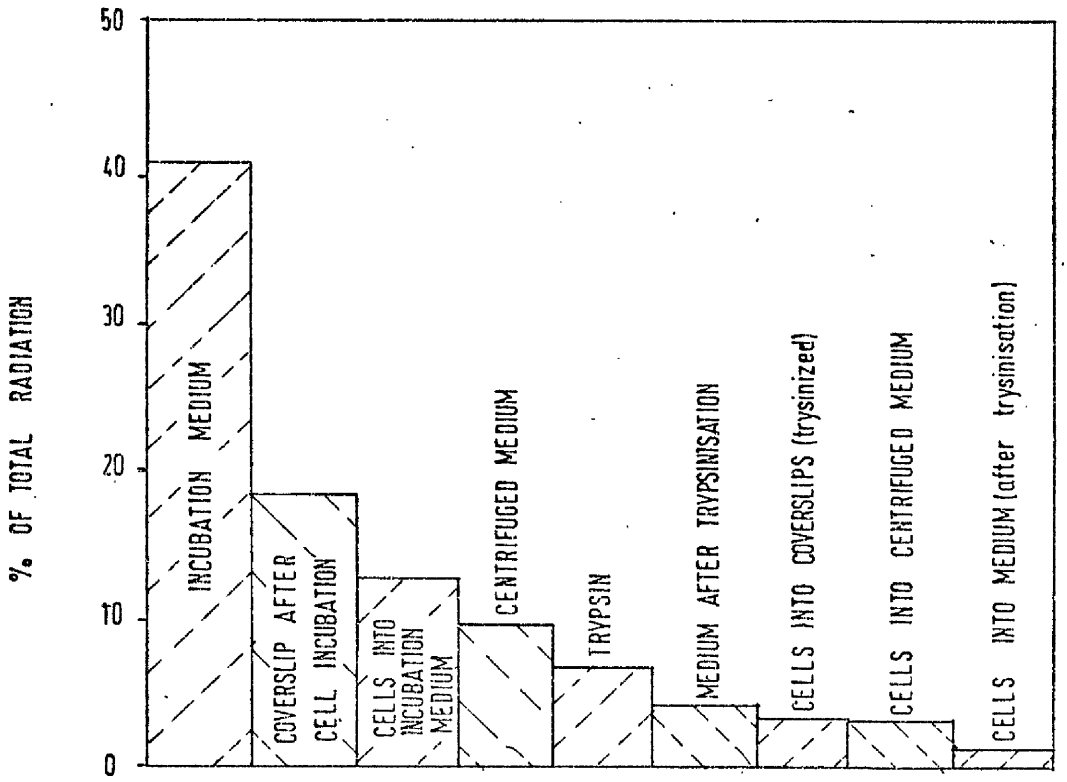


Fig 3.4 Distribution of radioactivity for C^{14} labelled DISTEAROYL LECITHIN coated glass surface (Unoriented). Results table 3.7, page 37.

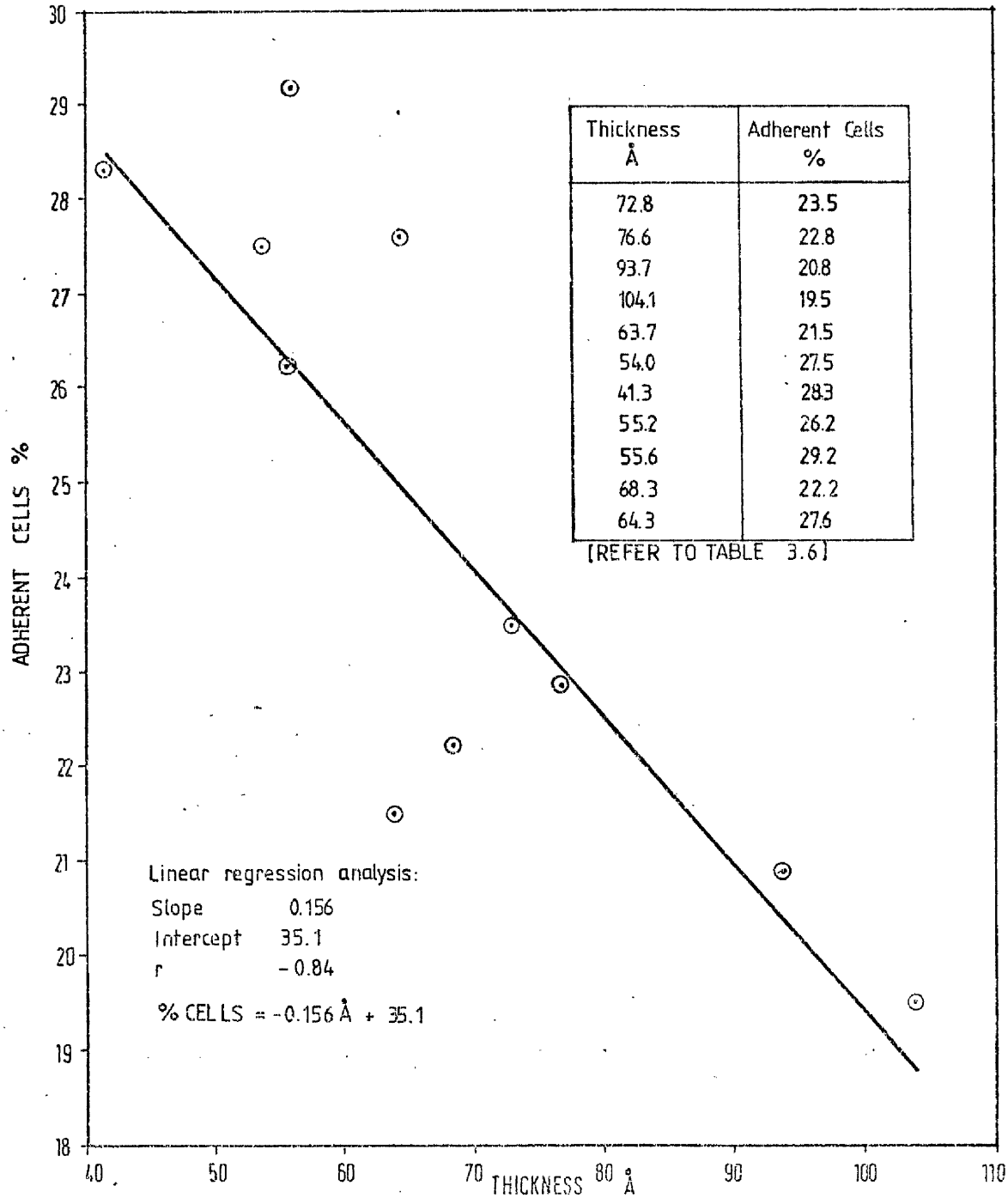
Table 3.6 - Cell adhesion on C^{14} labelled distearoyl lecithin coated glass surface (Unoriented)

No.	Distearoyl Lecithin μl	cpm	Thickness Å	Adherent Cells No.	Adherent Cells %
A	Background	30	-	-	-
	Control sample 5μl	13018	-	-	-
A1	Coverslip	4812	72.8	-	-
	After incubation				
A1	Coverslip	1444	21.8	35	23.5
B	Background	25	-	-	-
	Control sample 5μl	14515	-	-	-
B1	Coverslip	5648	76.6	-	-
	After incubation				
B1	Coverslip	668	9.06	34	22.8
C	Background	20	-	-	-
	Control sample 5μl	14043	-	-	-
C1	Coverslip	6686	93.7	-	-
C2	Coverslip	7427	104.1	-	-
	After incubation				
C1	Coverslip	2689	37.7	31	20.8
C2	Coverslip	1579	22.1	29	19.5
D	Background	24	-	-	-
	Control sample 5μl	19384	-	-	-
D1	Coverslip	6237	63.7	-	-
D2	Coverslip	5318	54.0	-	-
	After incubation				
D1	Coverslip	2428	24.7	32	21.5
D2	Coverslip	1869	18.97	41	27.5
E	Background	24	-	-	-
	Control sample 5μl	26336	-	-	-
E1	Coverslip	5531	41.3	-	-
E2	Coverslip	7381	55.2	-	-
	After incubation				
E1	Coverslip	3213	24.0	42	28.3
E2	Coverslip	2807	21.0	40	26.2
F	Background	25	-	-	-
	Control sample 5μl	26641	-	-	-
F1	Coverslip	7529	55.6	-	-
F2	Coverslip	9240	68.3	-	-
	After incubation				
F1	Coverslip	3181	23.5	44	29.2
F2	Coverslip	5051	37.3	33	22.2

Table 3.6 (continued)

G	Background	20	"	"	"
	Control sample 5µl	25510	"	"	"
G1	Coverslip	8338	64.3	"	"
	After incubation				
G1	Coverslip	4298	33.2	41	27.6

Area of petri dish (A) = 56.44 cm^2
Counting area of coverslips (a) = 0.0028 cm^2
Area of coverslip (C) = 3.3 cm^2
Total number of cells in dish (T) = 3.0×10^6
Settling time one hour at 38 C



Thickness Å	Adherent Cells %
72.8	23.5
76.6	22.8
93.7	20.8
104.1	19.5
63.7	21.5
54.0	27.5
41.3	28.3
55.2	26.2
55.6	29.2
68.3	22.2
64.3	27.6

[REFER TO TABLE 3.6]

FIG 3.5 Variation of percentage of cells sticking with thickness for C^{14} labelled DISTEAROYL LECITHIN coated glass surface. (Unoriented Film). Results table 3.6.

Table 3.7 - Distribution of radioactivity for C^{14} labelled distearoyl lecithin coated glass surface

(Unoriented film)

	Experiment Reference (Table 3.6)										Average
	C		D		E		F		G		%
	cpm	%	cpm	%	cpm	%	cpm	%	cpm	%	
Coverslip after cell incubation	1579	6.79	1869	13.31	2807	20.98	3181	22.27	4298	28.45	18.36
Cells into the incubation medium on filter paper	2886	12.4	1467	10.45	1626	12.12	2162	15.13	1953	12.93	12.61
Cells into the centrifuged medium on filter paper	93	0.4	33	0.24	143	1.07	102	0.71	2073	13.72	3.23
Cells into coverslip (tripsinized)	135	0.58	664	4.73	333	2.5	1072	7.5	194	1.28	3.32
Cells into medium (after trypsinization)	14	0.06	60	0.43	57	8.43	169	1.18	658	4.36	1.29
MEDIUMS EXTRACTED											
Incubation medium	8974	38.6	6677	47.54	5388	40.28	5803	40.62	5136	34.0	40.21
Centrifuged medium	5000	21.5	1111	7.91	2028	15.16	146	1.02	429	2.84	9.69
Trypsin	1712	7.36	1609	11.46	562	4.2	1337	9.36	269	1.78	6.83
Medium (after trypsinization)	2862	12.3	554	3.95	433	3.24	314	2.2	95	0.63	4.46
TOTAL	23255	100	14044	100	13377	100	14286	100	15105	100	100

3.6 THIN LAYER CHROMOTOGRAPHY

a) Cells in contact with lecithin surface

The amount of lecithin (distearoyl phosphatidyl choline) increased during film preparation on the glass surfaces. The microscopic observations showed that neural retina cells did not come in contact with the lecithin surface after one hour incubation in serum medium at 38°C on these very thick unoriented stearoyl lecithin films.
The floating cells in the incubation medium and the centrifuged medium were collected in order to measure incorporated C^{14} labelled phospholipid into the cells. A large amount of radioactivity was measured on the cells as given in table 3.IO and as shown on Figure 3.6.

The experimental work was carried on by extracting the incubation medium and centrifuged medium for thin layer chromotography in order to find chemical changes in the mediums. The observation of the T.L.C. results showed that the lysolecithin was released into the medium during incubation when the cells are in contact with lecithin surface. See Figures 3.6 and 3.7.

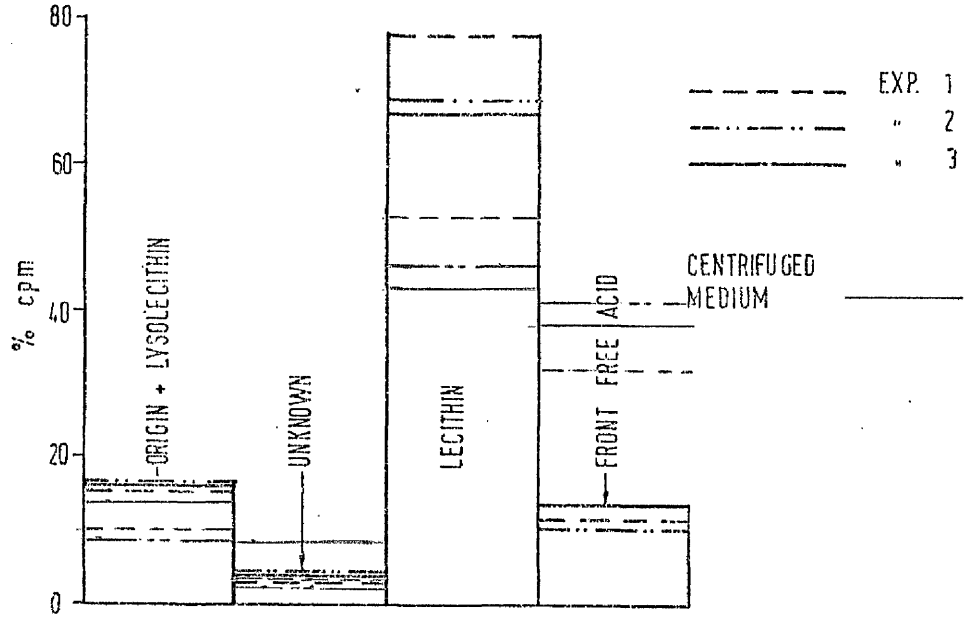


Fig 3.6 Distribution of radioactivity for cells in contact with lecithin surface.(Results table 3.I0)

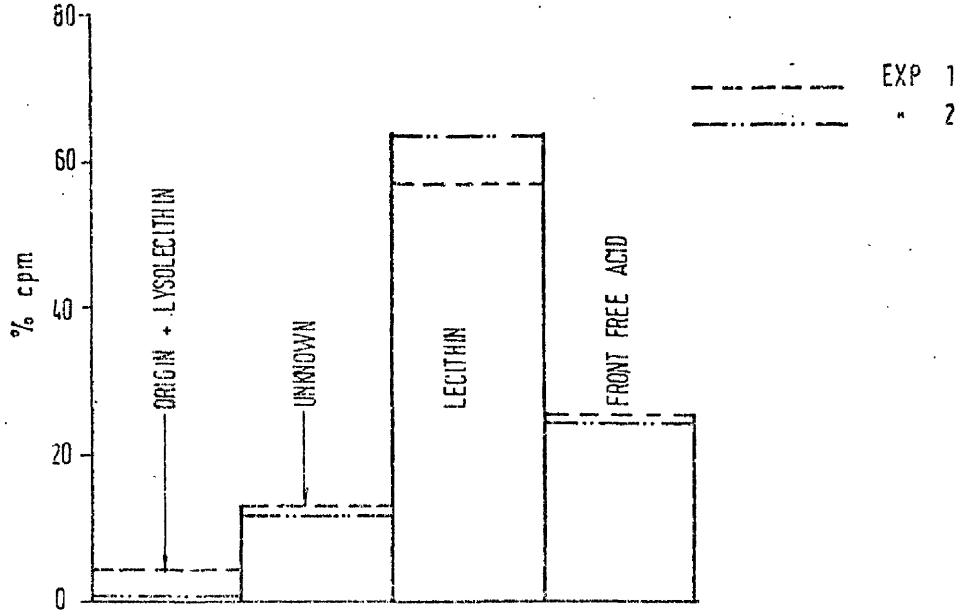


FIG 3.7 Distribution of radioactivity for cell uncontacted with lecithin surface.(Results table 3.I2)

Table 3.8 - Cells in contact with unoriented lecithin(distearoyl phosphatidyl choline) surface.

Distearoyl Lecithin	cpm	Thickness A
Background	20	
5 l control sample	31682	
Coverslip	28955	269
"	11629	108
"	12297	114
After cell incubation		
Coverslip*	10865	306
"	7469	131
After incubation in MEM without cell suspension		
Coverslip	6004	

Table 3.9 Percentage distribution of radioactivity (coverslip * in table 3.8)

Distearoyl Lecithin	cpm	% cpm	Adherent Cells %
Coverslip after cell incubation	IO 865	59.6	NO CELLS OBSERVED ON THE GLASS.
Cells into the incubation medium on filter paper	6 709	36.8	
Cells into the centrifuged medium on filter paper	656	3.6	
Mediums (incubation and centrifuged) were extracted for T.L.C.			

Table 3.10 - Cells in contact with lecithin (distearoyl phosphatidyl choline) surface (TLC)

No.	Lipid extracted from Incubation Medium TLC	cpm	% cpm
I	Origin + lysolecithin	2023	15.12
	Unknown	393	2.93
	Lecithin	9415	70.73
	Front, free acid	1549	11.58
2	Origin + lysolecithin	1170	16.65
	Unknown	336	4.78
	Lecithin	4809	68.46
	Front, free acid	710	10.11
3	Origin + lysolecithin	1363	16.09
	Unknown	341	4.02
	Lecithin	5638	66.53
	Front, free acid	1132	13.36

Table 3.11 - Cells in contact with lecithin (distearoyl phosphatidyl choline) surface

No.	Lipid extracted from Centrifuged Medium TLC	cpm	% cpm
I	Origin + lysolecithin	38	10.8
	Unknown	13	3.69
	Lecithin	188	53.41
	Front, free acid	113	32.1
2	Origin + lysolecithin	31	9.17
	Unknown	9	2.66
	Lecithin	158	46.75
	Front, free acid	140	41.42
3	Origin + lysolecithin	77	14.7
	Unknown	18	8.44
	Lecithin	288	43.51
	Front, free acid	201	38.36

b) Cells uncontacted with lecithin surface (TLC)

Comparable experiments were done with lecithin (distearoyl phosphatidyl choline) covered conical flasks. The cells were transferred into the flask and incubated for one hour in the shaker bath at 38 °C in order to prevent contact with lecithin surface. The results of T.L.C. showed that a smaller amount of lysolecithin released to the medium during incubation compared with the cells which were in contact with lecithin film. See Figure 3.7, page 39.

3.12 - Cells uncontacted with lecithin (distearoyl phosphatidyl choline) surface

No.	Distearoyl Lecithin	cpm	% cpm
	Background	20	
	5µl control sample	2800	
	Cells into the incubation medium on filter paper	10533	
	Extracted INCUBATION MEDIUM for T.L.C.		
I	Origin + lysolecithin	154	4.6
	Unknown	435	12.98
	Lecithin	1913	58.09
	Front, free acid	849	28.34
2	Origin + lysolecithin	31	0.72
	Unknown	482	11.35
	Lecithin	2710	63.8
	Front, free acid	1024	24.1

3.7 CELL ADHESION ON UNLABELLED DISTEAROYL LECITHIN AND DILINOLEOYL LECITHIN COATED GLASS SURFACE (LANGMUIR TROUGH METHOD)

Lecithin films on glass surfaces were prepared for both mono and multi-layer by dipping into unlabelled distearoyl and dilinoleoyl contained water surface by Langmuir method.

The results, as given in Tables 3.I3 and 3.I4 show that the percentage of adhesive cells on the lecithin surface do not show any relationship with the increasing lecithin thickness, however, a higher percentage of cells became detached compared with the clean glass surface. The cells kept their round form while being adhesive to the lecithin and the majority of them were individual.

Table 3.I3 Cell adhesion on unlabelled distearoyl lecithin coated glass surface.

No*	Barrier distance cm	Δgalvo reading	dyn/cm π	Thickness Å	T x 10^6	Adherent Cells	
						No	%
IA	I9	0.0	0.0	97.7	2.35	34	29.I3
2A	I9	0.3	0.9	97.7	2.I2	38	24.55
3A	I9	0.0I	0.0	97.7	3.0	4I	27.54
4A	I9	0.0	0.0	97.7	3.0	48	32.25
5A	I9	0.0	0.0	97.7	3.0	64	43.0
IB	9.7	8.22	25.7	49.4	2.35	39	33.42
2B	9.65	8.65	27.I	49.I	3.I2	47	30.36
3B	I0.75	8.35	26.I	54.7	3.0	44	29.56
4B	9.5	8.4	26.3	48.4	3.0	49	32.92
5B	9.3	8.6	26.9	47.3	3.0	32	2I.5
6B	9.95	8.8	27.5	50.6	3.0	I4	9.4
7B	9.0	5.6	47.6	45.8	3.0	26	I7.46

Area of dish (A) 56.44 cm^2

Counting area of coverslips (a) 0.0028 cm^2

Total number of cells in dish (T)

* IA-5A Surface pressure constant

IB-7B Surface pressure variable

Table 3.I4 Cell adhesion on unlabelled dilinoleoyl lecithin coated glass surface.

No*	Barrier distance cm	galvo reading	dyn/cm π	Thickness A	T x I0	Adherent Cells No	Adherent Cells %
AI	I9	4.70	I4.7	96.2	2.35	57	48.85
A2	I9	3.9	I2.2	96.2	3.I2	45	29.07
A3	I9	7.35	23.0	96.2	3.0	53	35.6I
A4	I9	5.8	I8.2	96.2	3.0	56	36.62
A5	I9	6.4	20.0	96.2	3.0	52	34.96
BI	I5.25	8.22	25.7	77.2	2.35	57	48.85
B2	I3.7	8.7	27.2	69.4	3.I2	46	29.7I
B3	I7.2	8.35	26.I	87.I	3.0	58	38.97
B4	I5.2	8.4	26.3	77.0	3.0	48	32.25
B5	I6.0	8.7	27.2	8I.0	3.0	5I	34.26
B6	I5.2	8.4	26.3	77.0	3.0	5I	34.26

Area of dish (A) 56.44 cm^2
Counting area of coverslips (a) 0.0028 cm^2
Total number of cells in petri dish (T)

* AI-A5 Surface pressure constant
BI-B6 Surface pressure variable

3.8 CELL ADHESION ON MONO AND MULTI-LAYER LECITHIN COVERED COVERSLIPS (BLODGETT TECHNIQUE).

The unlabelled distearoyl and dilinoleoyl lecithin films were transferred from the water surface to the glass surface by the Blodgett technique in the form of mono and multi-layers. The majority of the adhesive cells on the lecithin surface were individual and kept their round form.

The percentage of cells on the lecithin layer did not show any relationship with the dilinoleoyl thickness as shown in table 3.I5, however, less cells became adhesive compared with a clean glass surface. The distearoyl lecithin surface gives a similar result to that of the dilinoleoyl lecithin surface.

Table 3. I5 - Cell adhesion on mono and multi-layer lecithin covered coverslips

No.		T x 10^{6}	Thickness Å	Adherent Cells		
				No.	%	Average %
1	Dilinoleoyl Lecithin Dipped x 1	3.6	14.7	39	21.8	27.7
2		3.0		59	39.6	
3		3.0		37	24.9	
4		2.62		25	19.1	
5		3.25		42	26.1	
6		2.62		46	35.1	
7		3.6		49	27.5	
1	Dilinoleoyl Lecithin Dipped x 6	3.6	88.2	30	16.8	27.6
2		3.0		47	31.6	
3		3.0		52	34.9	
4		2.62		26	19.9	
5		3.25		50	31.1	
6		2.62		52	39.7	
7		3.6		34	19.1	
1	Distearoyl Lecithin Dipped x 1	3.6	14.7	36	20.2	21.3
2		3.0		35	23.5	
3		3.0		37	24.9	
4		2.62		20	12.4	
5		3.25		29	18.0	
6		2.62		35	26.7	
7		3.6		42	23.5	
1	Distearoyl Lecithin Dipped x 6	3.6	88.2	32	17.9	19.3
2		3.0		24	16.1	
3		3.0		40	26.9	
4		2.62		30	22.9	
5		3.25		45	15.5	
6		2.62		28	17.9	
7		3.6		32	17.9	

CHAPTER 4

D I S C U S S I O N

The result of the perfusion experiment shows that in a serum medium cell adhesion was strong to the untreated glass surface and the cells remained in round form while being adhesive to the film surface. When the glass surface was covered with a lecithin (distearoyl or dilinoleoyl phosphatidyl choline) film there was noticeable decrease in cell adhesion. The cell and glass interactions were affected by increasing the thickness of the film on the glass surface. It was observed that when the stearoyl lecithin surfaces were very thick few cells (table 3.6) or no cells (table 3.8) stuck to the lecithin on the unoriented film surfaces.

These observations can be interpreted to imply that a lecithin surface is less adhesive than glass and this appears to agree with Maroudas' (1973) result. In my experimental work it was observed that a large amount of C^{14} labelled phospholipid was taken into the cells when they were incubated in contact with the surface for one hour and lysolecithin was released into the medium. An experiment was carried out by shaking the cell suspension to prevent contact with lecithin contained glass surface and it was noticed that a smaller amount of lecithin was incorporated into the cell and less lysolecithin released. This fact suggests that contact is required for cell and lecithin interactions.

The adhesiveness of the cells at the glass surface must depend on the size of the contact area at the cell surface with glass interface. The cell contact may be governed by environmental

conditions and by surface wettability which will be dependent on the forces between molecules and the forces vary with the type of molecules. Glass and cell adhesion will occur if the surface areas can form multiple point at the contact area where the force of attraction bases take place. Ambrose's observation with surface contact microscope suggested that close contact within a few Angstrom units between a cell and its substratum may only exist over 1 percent of the total area of the cell opposed the substratum: But Curtis (1964/69) suggested that cell to glass contacts were much wider and once an adhesion has formed the cells depart from the spherical shape and the contact area presumably extends. When a cell area is opposed to the glass surface it might form short range adhesive bonds at 3.6 Å (Weiss) but Curtis (1967), Derjaguin (1960) indicated that the distances over which intermolecular forces act may be greater. The lecithin layers on the glass slide may be a simplified model of the cell membrane which cause cell-cell interactions with weak and loose adhesive bonds between the cell and film on the glass. Introducing zwitterionic lipid molecules such as lecithin on a glass surface may produce an electrostatic field on the glass which in turn affects lipid conformation. The density and sign of the film will depend on relative numbers, nature and localization of ionogenic groups of the lecithin layer on the glass surface. When the glass carries an adsorbed film beneath the cells there may be pentration of serum proteins into the film and these molecular interactions which depend

upon number of ionic and non-ionic bounding of the two different molecules. This complex layer on the glass surface can be considered to be more like a cell surface. The adsorption of various proteins on unimolecular films of charged lipid suggests that the primary association occurs almost entirely between the charged ionic groups of the interface and the protein (Matalon and Schulman 1949). A cell and glass attachment may be governed by adsorbed serum proteins and proton penetration from the suspending medium and attachment of the cations could affect adhesion (Rappapot et al 1960)The result of the experiments showed that a smaller amount of cells were adhered to lecithin compared with glass in serum medium when they were in contact. This might be due to the degree of hydrophobicity or hydrophilicity of the lecithin layer on the glass surface.

The incorporation of lipid in the cell may result from a number of pathways having their origins in protein metabolism. If cell surface proteins have the opposite charge to the lecithin layer this will cause the adsorption until they have the same sign as the opposed surface, suggesting an electrostatic interaction between charged groups. On the other hand another possibility may be that the lecithin is taken in phagocytosis. It is known that a cell with carboxyl groups on their membranes are able to adhere in a non-specific way by the rate of Ca^{++} ions which can form a bridge between the negative charges on the cell peripheri proteins and negative charge of the serum proteins. Another explanation arises in the following manner. It was noticed that much less lysolecithin was released and less labelled lecithin incorporated into the cells

when suspension was incubated in a shaker bath in order to prevent cell contact with glass surface (see Figure 4.I). This suggests that contact of a cell with a lecithin surface is necessary for hydrolysis of the lecithin and thus the phospholipases involved in production of lysolecithin can not be released by the cells. Therefore, it is clear that contact is required for their interaction. Since lysolecithin is known to diminish the adhesion of cells I can explain the observation that cells will not adhere to lecithin as being due to conversion of this to lysolecithin when they contact a lecithin surface. This explanation for non-adherence of cells is different from that stated by Maroudas (1973) who suggested that cells will not adhere to a hydrophobic surface with a low yielding stress under a transverse load and lecithin is an example of a hydrophobic surface with low yield. Ivanova (1973) suggested that it is not clear whether the ionogenic characteristic of the surface of lipid film is essential for its non-adhesiveness and she supports Liberman who suggested that electronegativity of the surface phospholipid film may play an important role in adhesion. The adhesion of the neural retina cells on the clean surface were strong and promoted by serum. This must have been based on consideration of the hydrophilic nature of the glass surface which was predominantly wettable. The cells will adhere to thin films either by lysing them away or because little lysolecithin can be formed. Therefore, Maroudas' explanation appears to be unnecessary. The experimental observations also showed that the spreading could not occur on the lecithin layer and the lesser amount of adhesive cells adhere on it compared with clean glass.

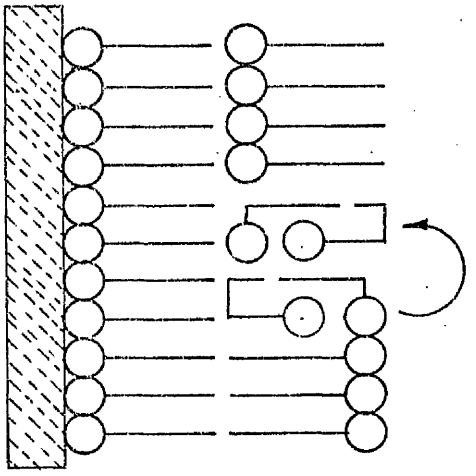


Fig 4.I Langmuir's overturning mechanism.

The transfer of the monolayers to a solid glass by dipping technique or floating may produce different type of films such as X type films which are hydrophobic, as dipping goes on more and more irregular structure will form in the case of X type films on mixed X/Y type films. This structure probably enhances the overturning (see Figure 4.1). This sort of structure might effect the binding of the serum proteins to the lecithin layer and might reduce the amount of serum adsorption to the lecithin surface. The serum proteins adsorbed on the interface of the layer are probably fully unfolded and this may reduce the potential at the interface affecting interaction with the lecithin surface before lysolecithin is released by the cells. The result of physico-chemical forces acting between the cell surface, substratum and the intervening medium must effect the physiological response of the cell to contact with surface and any variation in them would certainly be reflected

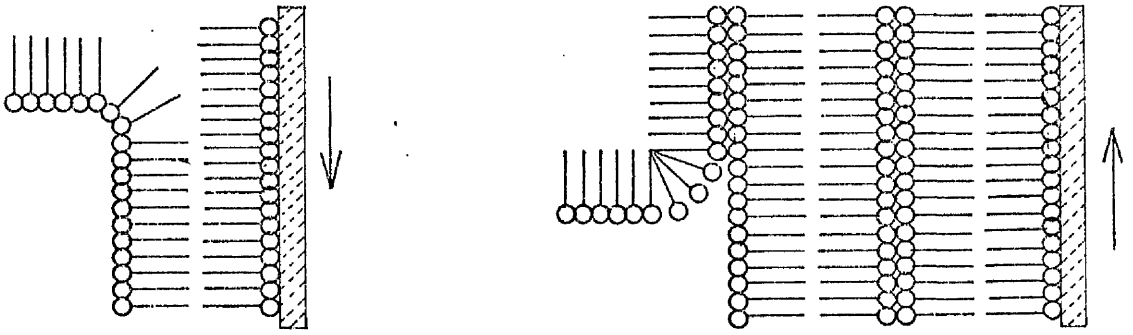
in altered or modified behaviour of the cell and the spreading process. After lecithin incorporation into the cell the released lysolecithin affects the cell interactions or lecithin is incorporated as lysolecithin by the cell. The enzymic reaction demonstrated at the phospholipid-water interface by Dawson indicated that lysolecithin will leave a unimolecular film of lecithin at the air-water interface when this is digested with phospholipase A. The French workers E.La Breton and J.Pantaleon(1958) and later J.Etienne (1966) and J.Polonowski (1963) first noticed a connection between phospholipid hydrolysis and cholesterol ester formation in the plasma. They suggested that fatty acid released from the phospholipid, phosphatidyl choline by phospholopases were subsequently incorporated. A study of the digestion of unimolecular films of ^{32}P-lecithin and ^{32}P-phosphatidyl ethonolamine by cobra venom phospholipase A indicated that phospholipids were hydrolysed without the necessity for any activating agent and that the lysophospholipids released by the action largely left the film and entered the bulk phase.

During lecithin incorporation into the neural retina cells the fatty acid composition of the phosphoglycerides in a membrane might alter. Any distribution in chain length of phospholipids and also their degree of unsaturated fatty acid chain may cause to effect on membrane fluidity (Curtis). The incorporated lipid molecule probably will associate with other types of lipids in the cell and will combine with protein to form lipoprotein structure. This might change the specific combination of the cell lipids and cell proteins and probably will cause the stimulation of enzymic activities.

The chain length may reflect a precise requirement for the stereo chemical orientation of enzyme-substrate complex. It is known that phospholipases in the cell membranes are either phospholipase A_1 or A_2 or probably sometimes both, which can release the fatty acid from the 1 position and also the 2 position. Therefore, phospholipases have an important role in reducing the micelle size removing accumulated phospholipids. Metal ions such as Ca^{++} , Mg^{++} may be retained as an intricate or essential part of lipid protein complex. Dawson and Hemington (1967) had pointed out that in the presence of calcium, little enzymic adsorption occurred on a pure lecithin surface and Mg^{++} also prevented the denaturation of enzyme. Probably after lecithin incorporation these cations play a highly specific role in the further formation or breakdown of enzyme-substrate complex. De Haas (1966) discovered that phospholipases activation is due to cleavage of a small peptide from protein chain by a proteolytic enzyme. Curtis suggested that incorporation of saturated fatty acids take place in R_2 position and show a rise in adhesiveness, incorporation of unsaturated fatty acid cause to fall in adhesiveness.

The result of the adhesiveness of neural retina cells on C^{14} labelled distearoyl lecithin and unlabelled dilinoleoyl lecithin coated glass surface by Blodgett technique and the result of the adhesiveness of these cells the unlabelled lecithin (distearoyl and dilinoleoyl) coated glass surface by Langmuir technique did not have the value of adhesiveness that is expected. These experimental results did not show a clear relationship with the increased thickness of the lecithin layer on the coverslips. A general conclusion seems to

be that the lipid is not in a fully condensed condition and that the lipid also is not in too much of a fully expanded condition as shown in figure 4.3. The lecithin film transferred by the Blodgett technique to the surface will reflect the degree of flexing and twisting of the hydrocarbon chains on the air/water interface. Therefore,at the low compression the molecules are



a) Equal Thickness

b) Variable Thickness

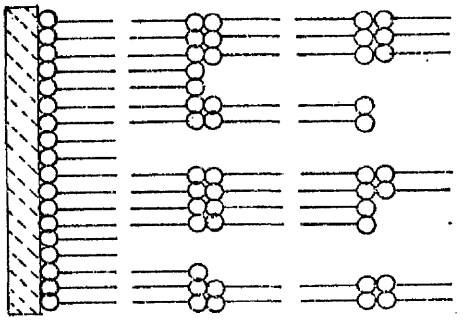
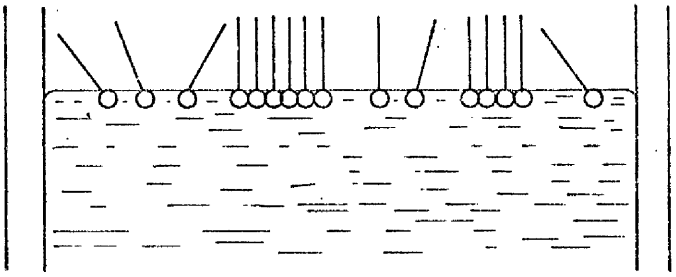


Fig 4.2 Formation of multilayer films on glass surface.

orientated at different angles or form packed aggregates. The shape of the hydrocarbon chain and less condensation of the dispersed lecithin on the water surface will be a significant factor which can be thought to cause differentiation of the lecithin packing on the glass surface. The other possibility is the form of the molecular organisation of the lipid layer on the glass surface.

The lecithin molecules can arrange themselves in many ways on top of the first layer for every irregular dipping of the glass into the water (see Figures 4.I,4.2,4.3). These different organisations of the lecithin molecules will either decrease or increase the thickness of the layer with the length of number molecules so that the layer will organise in different thicknesses on the glass surface. The formation of model membranes may also be

a) Low compressiom



b) High compression

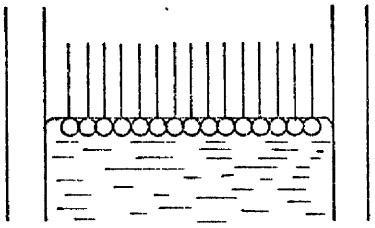


Fig 4.3 Diagram of monolayer films on water surface.

related to the degree of fluidity of the hydrocarbon chains of the lipid at the temperature of the experiment. For example,it was noticed that fully saturated phospholipids do not form myelin figures at room temperature(Chapman et al I967). For these reasons it can be assumed that the measurement of the adhessiveness of the cells will show differentiation for different areas on the same coverslip by changing lecithin thickness. These techniques do not indicate much about adhesion to lecithin because much of the glass surface was free of lecithin unlike the situation with the unoriented films.

Rosenberg suggested (I962) that the interactions between tissue culture cells and multi-molecular layers are functions of the number of monolayers which are underlying the cells. He showed that the attachment and spreading of cells was increasingly lengthened when he increased the number of subjacent monomolecular layers of barium stearate-stearic acid on the quartz slides by the Blodgett Technique. He predicted that if a random population of cells were grown on substrate of varying thickness a statistically significant sample should migrate and be entrapped in the lower regions of the layer and even on a chrome plated glass surface if the thickness reaches down to the glass surface, to which cells adhere more readily.

There have been few reports concerning lecithin surface which is believed to play a crucial role being a non-adhesive surface for cell adhesion. O. Yu Ivanova suggested that the phospholipid is the only film which is a non-adhesive surface for experiments with fibroblast and other types of culture cells (1973). In her experiment with time-lapse cinematography she used mouse embryo fibroblast, the cell of L strain, Chim strain cells and epithelial kidney cells. Her results indicated that on the lecithin coated glass surfaces those types of cell did not spread but retained their spherical form for many hours continuously formed and withdrew short cytoplasmic process and the same result was obtained in my experimental work with neural retina cells except for the cytoplasmic process. She showed the non-adhesiveness of the lecithin by further experiments which was slightly different than mine. When she covered a part of the glass by lipid layer and the cells oriented themselves parallel to film edge and they did not stop undulating but did not attach to the phospholipid. She proved that the characteristic of the surface of the film was non-adhesive, by cell migration to the narrow scratched regions on the lecithin layer after the coverslip was incubated with cells. This notion is also supported by Maroudas' observation that fibroblast cell did not attach to the layer but grew in tracks scratched through on the lecithin layer in medium containing serum at 37 ° C. Therefore, the attachment of a cell such as non-adhesive surface must have been by mediated serum proteins in the medium.

Curtis' experiment is with neural retina cells in a medium containing CoA, ATP and oleate showed that the adhesiveness of the cells were

maintained in a such medium and the fatty acid was rapdily incorporated into the plasmalemmal phospholipids and the other components of the cell surface. Some sort of rapid lecithin incorporation occurred when the neural retina cells were incubated on the lecithin layer in serum contained medium during my experiment. Curtis' treatment of the cells with phospholipase A and incubation of cells with lysolecithin led to diminution in cell adhesion. Therefore, the fatty acid composition of tissue lipids can be suited to the requirements of the cell and phospholipases can become an important enzyme in the regulation of lipid composition and adhesion.

Curtis indicated that unsaturation and reduction of chain length would be expected and did reduce electrodynamic forces and these might reduce intramembrane force of attraction and intramolecular forces (affecting plasmalemmal fluidity)

Research of contact interaction of RPMI No. 41 cells and Mastocytoma cells with glass has been carried out by Weiss. During his experiment the RPMI No. 41 cells became strongly contacted to the glass. He suggested that the adhesiveness of these cells to the glass were in primary minimum because the cell overcame the electrostatic potential barrier by producing protrusions from the cell when they dislodged from the glass they left material on the surface. With this microruptures from the cell periphery he also proved that the total adhesive bonds between a cell and glass have greater energy than the total adhesive bonds holding the cell periphery together.

Deryagin, Landau, Verwey and Overbeek considered the interaction

between particles that would take the place of electrostatic forces of repulsion acted together with the London forces of attraction. Curtis pointed out that in the Verwey and Overbeck treatment of colloidal stability it is predicted that there will be regions of adhesion stability due to dispersion forces at particle separation of the order of 100 - 150 Å, and that an electrical potential barrier makes close approach difficult. He calculated that the adhesive energy for BHL21/CB and L929 and chick neural retina cells which he showed were adherent in secondary attractive minimum within molecular contact with the separation of 100 Å. He indicated that the cell could form stable adhesion in secondary attractive minimum when separated by 100 - 150 Å.

However, Weiss states it would appear extremely unlikely that the interaction of this type would provide enough attractive energy to stabilise tissue system. Weiss suggested that the two different cells have the same mean electrostatic mobilities such as RPMI No. 41 and Mastocytoma cells, one of them can form adhesive in primary minimum while the other became adhesive in secondary attractive minimum with glass surface. He concluded that the contact with other cells or non-cellular surface charged group depend on their presence and spatial location at the surface. Pethicia suggested that if all membrane are held to each other at the separation 150 Å actual separation will be smaller than that and mediated by forces additional to the dispersion forces and the size of the electrical potential barrier will not prohibit the close approach if contact made at protuberances. He indicated that membranes can make stable adhesion with the 5 Å separation in a weak

dispersion force by Ca^{++} bridging. Armstrong and Jones emphasised that divalent cations most effective in promoting cellular adhesion vary from one system of cells to another. A pertinent example is the contrast between their own results with amphibian epidermal cells which show that the Ca^{++} ion is most effective in preventing dissaggregation, whereas Armstrong had earlier demonstrated that the Mg^{++} ion is most effective in bringing about reaggregation of the cells of dissociated limb buds of chick embryos Alison (1963) indicated that the divalent or trivalent cations affect cell adhesion perhaps by bridging from one cell to another.

Danielli (1951) stated that Ca^{++} would be bound to the carboxl group of the proteins and phosphate groups of lipids. Garvin (1961) suggested that a receptor molecule which could undergo a conformational change on combining with the metal ion leads to increased adhesiveness. A second alternative is that a metal activated enzyme is crucial to the adhesive process. Curtis proposed that the primary role of the positively charged divalent cations in promoting cell adhesion was to lower cells' net negative surface charge.

The effectiveness of polyvalent cations on the cell adhesion is clear . will cause the reduction of the forces of repulsion over a range of separations and may permit adhesion to form. The potential energy barrier probably must have been surmounted before the surfaces are close enough to bind polyvalent cations. Brownian motion may alternatively provide to approach two surfaces at large distance where they need enough energy to overcome repulsion forces and link the polyvalent cations to the polar side of the cell membrane

proteins and opposed surface. Therefore, it would appear that close range bridging agents may be important as a direct linkage between the cell and the glass interface.

In my experimental work the charge distribution at the neutral retina cell surface may be hetrogeneous and non-polar regions of the cell surface may be involved in contact interactions by releasing lysolecithin to the medium leading to decreased adhesion on the lecithin surface in serum contained medium. It is probable that the decreased adhesiveness of this cell on a lecithin surface is a consequence of lysolecithin release but it is clear that we do not yet have a full understanding of all the physico-chemical forms between cell substrate and environment.

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