STUDIES OF TRANSPORT THROUGH CURVED AND PLANAR LIPID BILAYERS

by

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TABLE OF CONTENTS

SUMMARY

This study is concerned with bilayer lipid membranes (BLM), of bimolecular thickness, as simple potential models for some aspects of the physical chemistry of biological membranes.

Lipid bilayers can be assembled from lipids either as spherical structures, liposomes, which are layer lattices of alternating, closed bimolecular lipid sheets intercalated by aqueous space, or as single planar structures that separate two aqueous phases. These models, while having both advantages and shortcomings, complement each other, and both types of systems have been considered here.

The first section of this work deals with the effect of electric fields on liposomes prepared from only lipid components. Several methods of preparation, such as sonication, removal of detergent from mixed lipid/detergent micelles, and extrusion through polycarbonate membranes, have been investigated. Detailed characterisation of each system was undertaken to determine the method resulting in the most suitable liposomes. Effects of applied fields have been linked to changes, occurring in the arrangement of the lipid molecules constituting the bilayer, which affect the permeability of the membranes. In this work a solute was trapped in the internal volume of the liposomes and fluorescence or absorption spectroscopy was used to detect its presence in the external medium after the application of a field.

In the second section the movement of electrons across the lipid bilayer and redox reactions at the membrane/solution interfaces has been investigated. Planar bilayer films allow easy access to the aqueous phases on both sides of the bilayer so that electrical properties, such as specific

capacitance and resistance, can be measured. Experimental apparatus consisted of two monolayer troughs separated by a septum containing an aperture. A lipid monolayer is applied to the aqueous phase on either side of the septum. When the level of aqueous solution in each compartment is raised above the aperture, a lipid bilayer membrane is formed by the apposition of the hydrocarbon chains from each monolayer. This method results in membranes that are almost free of entrapped solvent, with the added advantage of enabling the formation of asymmetrical lipid bilayers.

Investigations of the electrical properties of such membranes have been undertaken and results compared to those membranes containing more solvent, the black lipid films. Preconditioning of the membrane support, an essential prerequisite for the formation of stable membranes, has also been considered, as well as the stabilising effect observed with the addition of cholesterol to the BLM-forming solution.

The liquid-crystalline structure of BLM is a very good insulator, but incorporation of electroactive modifiers, such as tetracyanoquinodimethane (TCNQ), ferrocene, and iodine, cause marked changes in the electrical properties when redox couples are present in the aqueous solutions on either side of the membrane. The modified membranes act as electronic conductors with reduction occurring on one side and oxidation on the other side of the interface. Cyclic voltammetry was used to investigate electron transfer and redox reactions at the membrane/solution interface of suitably modified membranes, using several redox systems. The resulting voltammograms, confirming that the modified BLMs act as the working electrode, indicated that electron transfer was indeed occurring across the membranes.

STATEMENT

This thesis contains no material which has been accepted for the award of any other degree or diploma in any other university and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text of this thesis.

I consent to this thesis being made available for photocopying and loan.

KAREN E. CONNELL

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ABBREVIATIONS

AC	alternating current	M.W.	molecular weight
BLM	bilayer (bimolecular) lipid membrane	MWC	molecular weight cutoff
		nm	nanometre
C	capacitance	NMR	Nuclear Magnetic Resonance spectroscopy
cm	centimetre		
CMC	critical micelle concentration	psi	pounds per square inch
DC	direct current	q	charge
dm	decimetre	QELS	quasielastic light scattering
E	voltage		
F	farad	R	resistance (or $8.314 JK^{-1}mol^{-1}$)
$FATMLV_S$	frozen and thawed multilamellar vesicles	rpm	revs per minute
		SUV	small unilamellar vesicle
g	gram	SUVET	small unilamellar vesicles by extrusion techniques
Hz	hertz		
i	current		
i.e.	that is	t	time
kPa	kilopascal	T	temperature
LUV	large unilamellar vesicles	T_m	phase transition temperature
$LUVET_S$	large unilamellar vesicles by extrusion techniques		
		v/v	volume (cm^3) per volume (cm^3)
M	molar		
mg	milligram	V	volts
ml	millilitre	W	watts
MLV	multilamellar vesicle	w/v	weight (g) per volume (cm^3)
mm	millimetre		
mol	mole	w/w	weight (g) per weight (g)
mV	millivolts		

CHAPTER 1 - INTRODUCTION

Knowledge of the boundary or barrier which separates the inner contents of the cell from its surroundings is believed to be central to an understanding of life processes. It has been accepted for many years that the basic structural component of biological membranes is a bimolecular lipid layer modified by oil- and water-soluble proteins. Research has shown that the physical properties of bilayer lipid membranes separating two aqueous phases are similar to the corresponding properties of natural membranes [1,2,3,4]. Therefore bilayer lipid membranes, even without the protein components known to be present, are valid models for investigations of some aspects of biological membranes.

Membrane lipids are amphipathic molecules, consisting of non-polar, long hydrocarbon chains at one end, while the other end of the molecule is highly polar and therefore has a different reactivity to water. In an aqueous environment, lipids adopt specific arrangements due to the presence of the non-polar chains and polar headgroups in the same molecule. Examples of the polymorphic phases of lipids in aqueous solution are shown [Fig. 1.1]. The orientation of the amphipathic lipids to form micelles, inverted micelles, or lamellar (smectic) phases occurs spontaneously, requiring no input of energy. The polar regions of an amphipathic lipid form hydrogen bonds with water molecules, which compensate for the distortion of pure water occurring with the addition of lipids to an aqueous phase. As non-polar groups form no hydrogen bonds with water, lipids are thought to increase the ordered structure of water as the hydrogen-bonding between neighbouring water molecules increases. Thermodynamically, this causes a decrease in the entropy of the system and

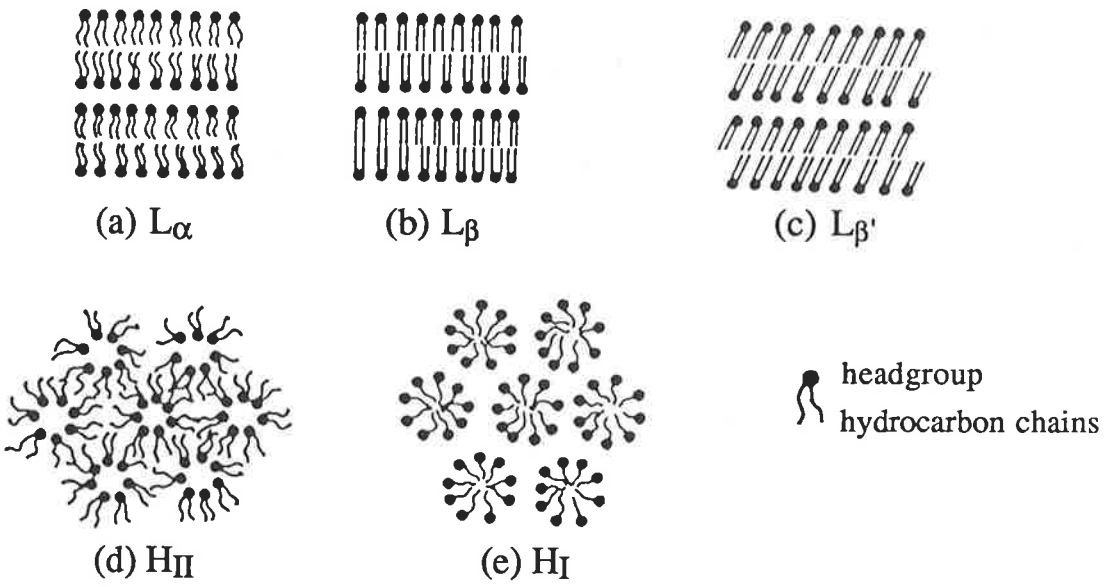


FIGURE 1.1 : A schematic representation of several lipid polymorphic phases. The L_α phase is a liquid crystalline (melted chain) bilayer phase, whereas in the L_β and $L_{\beta'}$ phases the chains are in a gel (frozen) state. The H_{II} and H_I phases consist of hexagonally packed tubes of liquid crystalline lipids [5]. (Each lipid molecule consists of a polar headgroup and a non-polar hydrocarbon chain.)

this unfavourable situation is remedied by burying the non-polar groups away from the aqueous phase. Bilayer assembly is predominantly due to this hydrophobic effect [6]. The geometry actually occurring in equilibrium is one in which compromises are made between entropy and all intermolecular interactions, such that the overall free energy per molecule is minimised. Factors to be considered include Van der Waals forces, hard core molecular packing constraints, hydrogen bonding, and electrostatic and hydrophobic interactions [7]. Lipids that form bilayers are those that cannot pack into small micellar structures, because i) the non-polar hydrocarbon chains are too bulky to fit into such small aggregates while maintaining a surface area at the optimal value, which is the case when the lipids contain two or more hydrocarbon chains; ii) a larger area is needed to accommodate their polar head groups [8,9]. The "optimal surface area" per head group, at which the

total interaction free energy per lipid molecule is a minimum, is a result of the two opposing forces, the hydrophobic force tending to decrease, and the hydrophilic one tending to increase, the head group area in contact with water [9]. Under certain conditions, for example, in the presence of excess water, it becomes more favourable for closed spherical bilayers, liposomes, to form rather than infinite, planar bilayers. Mesophases that lipids spontaneously adopt when dispersed in water are therefore the result of forces arising from intrinsic factors such as, the nature of the lipid head group, length and degree of the unsaturation of the lipid chain(s), or extrinsic factors such as, the lipid concentration, hydration temperature, pH, ionic strength, divalent cations, or the presence of other lipids, ions, or proteins [10]. The preferred structural forms of some lipids are:

Micellar phase - lysophospholipids, detergents

Bilayer phase - phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), sphingomyelin

Hexagonal phase (H_{II}) - phosphatidylethanolamine (PE) (unsaturated), PS (pH<4), cholesterol

Non-bilayer lipids, such as PE and cholesterol, can be stabilised in a bilayer structure by the presence of bilayer-preferring lipids, for example, PC, PS, or sphingomyelin. It has been found that 20-30 mole percent of bilayer-preferring lipids are required to maintain a bilayer organisation when mixed with H_{II}-preferring lipids [11]. The stabilising effect of some common membrane lipids increases in the following order:

sphingomyelin < egg yolk lecithin < PS < PE < cholesterol [12].

Pure phospholipid bilayer membranes undergo a phase transition, upon heating, from a solid "gel" (L_β) phase to a fluid "liquid-crystalline" (L_α) phase. This phase transition is accompanied by the following changes:

i) increased conformational freedom and flexibility of the hydrocarbon chains leading to decreased segmental orientational order and decreased bilayer thickness, and

ii) increased lateral diffusivity of the lipid molecules parallel to the plane of membrane and the onset of a more rapid rotational diffusion about the long molecular axis, corresponding to decreased microviscosity.

The higher fluidity of the high temperature phase is therefore caused by changes in both orientational order and microviscosity. On heating, crystalline lipids pass through an intermediate mesomorphic or liquid-crystalline phase before becoming fully liquid. The temperature at which this crystalline to liquid-crystalline transition occurs involves rearrangements in the bilayer form and is dependent on factors such as, the fatty acid composition, the type of polar head group, the presence of water, and the addition of other lipids. The hydrocarbon chain length is a major determinant for T_m, the phase transition temperature. Increasing the number of segments on the hydrocarbon chains increases T_m. Conversely, increases in the chain tilt and degree of saturation tend to decrease T_m. It is important that the temperature is monitored throughout experiments involving lipids, so that their state is always known and can be considered when interpreting the results.

Cholesterol, a neutral lipid, is often distributed randomly in biological membranes. It is known to influence the packing of hydrocarbon chains of phospholipids, possibly by forming a cholesterol-phospholipid complex. *In vitro* studies with liposomes and bilayer films indicate that the presence of cholesterol in the membrane reduces the permeability and

increases the stability of the structure [13]. Spectroscopic studies indicate that cholesterol increases the hydrocarbon chain mobility in lipids in the gel state at temperatures below their phase transition, and reduces chain mobility in lipids at temperatures above their phase transition. At sufficiently high cholesterol concentrations, the gel-liquid crystalline phase transition is completely eliminated in cholesterol/phospholipid mixtures and the system has the properties of a two-dimensional liquid over a wide range of temperatures [14]. Cholesterol has been added to some of the bilayer lipid forming solutions used in this work to increase the stability of the membranes.

The formation of bilayer lipid membranes (BLM) of bimolecular thickness *in vitro* enables the systematic study of membranes under well-defined and easily controlled conditions. Studies of these much simpler model membrane systems can give valuable basic information on the physical behaviour of membrane components and the use of progressively more sophisticated, well-characterised systems can then give additional insight into the roles that these structures may play *in vivo* .

Studies of lipid bilayers, in the form of planar structures and liposomes (the closed spherical bilayers) have increased the understanding of the behaviour of cell membranes.

Liposomes are layer lattices of alternating bimolecular lipid sheets, intercalated by aqueous spaces [Fig. 1.2]. During preparation of liposomes, where the dry lipids are hydrated with aqueous solutions, there is opportunity for solutes to be sequestered in the internal aqueous space. While much research has concentrated on the possibility of using liposomes to administer drugs, they are also useful in permeability studies. Increasing concern about the effects of electromagnetic fields on humans, has prompted investigations into their effects on membranes. Applications of fields to

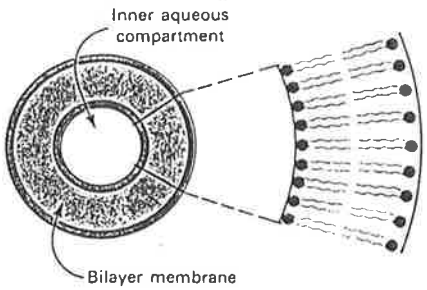


FIGURE 1.2 : Diagram of a lipid vesicle.

membranes have caused changes, occurring in the arrangement of the lipid molecules constituting the bilayer, which affect their permeability. In Part I liposomes were used, as simplified models for biological membranes, in further investigations of these field effects. A solute was trapped in the internal volume of the liposomes, and spectroscopic techniques, fluorescence and absorption, were used to detect its presence in the external medium after the application of a field.

In Part II Montal-Mueller planar bilayer lipid membranes were used as model systems for studying the electrical properties of lipid bilayers. This type of membrane is particularly useful as it allows easy access to the aqueous phases on both sides of the bilayer. Experimental apparatus consisted of two monolayer troughs separated by a septum containing an aperture. A lipid monolayer is applied to the aqueous phase on either side of the septum. When the level of aqueous solution in each compartment is raised above the aperture, a lipid bilayer membrane is formed by the apposition of the hydrocarbon chains from each monolayer. This method results in membranes that are almost free of entrapped solvent. They have the further advantage of facilitating the formation of bilayers that exhibit phospholipid asymmetry, which is characteristic of many biological membranes [15,16,17,18,19]. Studies have been made of the electrical properties of the membranes formed and the electron transfer across the membrane has also been investigated.

REFERENCES

1. C. Huang and T. E. Thompson: *J. Mol. Biol.*, 13 , 183, (1965).

2. A. D. Bangham, M. M. Standish and G. Weissman: *J. Mol. Biol.*, 13 , 253, (1965).

3. C. Huang and T. E. Thompson: *J. Mol. Biol.*, 15 , 539, (1966).

4. A. D. Bangham, M. M. Standish and J. C. Watkins: *J. Mol. Biol.*, 13 , 238, (1965).

5. S. M. Gruner, P. R. Cullis, M. J. Hope and C. P. S. Tilcock: *Ann. Rev. Biophys. Biophys. Chem.*, 14 , 211-238, (1985).

6. C. Tanford: "*The Hydrophobic Effect : Formation of Micelles and Biological Membranes* ", John Wiley and Sons, New York, (1980).

7. Sol M. Gruner: in "*Liposomes : From Biophysics to Therapeutics* ", (Marc J. Ostro, ed.), Marcel Dekker, Inc., New York, (1987).

8. J. N. Israelachvili: in "*Physics of Amphiphiles : Micelles, Vesicles and Microemulsions* ", (V. Degiorgio, and M. Corti, eds.), North-Holland Physics Publishing, Amsterdam, (1985).

9. Jacob N. Israelachvili, D. John Mitchell and Barry W. Ninham: *Biochim. Biophys. Acta*, 470 , 185-201, (1977).

10. Colin P. S. Tilcock: *Chem. Phys. Lipids*, 40(2-4), 109, (1986).

11. Pieter R. Cullis, Michael J. Hope and Colin P. S. Tilcock: *Chem. Phys. Lipids* , 40(2-4), 127, (1986).

12. R. Schubert, K. Beyer, H. Wolburg, H. Jaroni and K. Schmidt: in "*Liposomes as Drug Carriers* ", Symposium Tübingen, October, 1984, (K. H. Schmidt, ed.), Georg Thieme Verlag, Stuttgart, (1986).

13. M. G. Rumsby: in "*Companion to Biochemistry. Vol. II : Selected Topics for Further Study* ", (A. T. Bull, J. R. Lagnado, J. O. Thomas, and K. F. Tipton, ed.), Chapter 6, Longman, London, (1979).

14. Myer Bloom and Ole G. Mouritsen: *Can. J. Chem.*, 66 , 706, (1988).

15. M. D. Houslay and K. K. Stanley: "*Dynamics of Biological Membranes* ", Wiley, Toronto, (1982).

16. J. A. Higgins and C. A. Pigott: *Biochim. Biophys. Acta* , 693 , 151-158, (1982).

17. D. M. Michaelson, G. Barkai and Y. Barenholz: *Biochem. J.*, 211 , 155-162, (1983).

18. J. A. F. Kamp: *Annu. Rev. Biochem.*, 48 , 47-71, (1979).

19. G. P. Miljanich, P. P. Nemes, D. L. White and E. A. Dratz: *J. Membrane Biol.*, 60 , 249-255, (1981).

PART I - LIPOSOMES

CHAPTER 2 - INTRODUCTION

As advancing technology plays a major role in modern society, our everyday exposure to electric, magnetic, and electromagnetic fields has increased. This is of great concern and has prompted research into the possible effects of such exposure on human health.

The biological effects of microwave radiation has received much attention, particularly at a frequency of 2450 MHz, which is used extensively in therapeutic, domestic, and industrial applications, and is proposed as a transmission frequency for future power satellites [1]. Three conclusions arising from such studies are particularly important. Firstly, exposure of rats to low intensity 2450 MHz [2] (or 2800 MHz [3]) magnetic fields has resulted in changes in their behaviour [2] with a pronounced effect under *pulsed* rather than *continuous* wave exposure [3]. Secondly, the *frequency* of radiation is highly relevant as was shown by the finding that cardiac activity of isolated chick embryo hearts was affected by 2450 MHz microwave radiation [4], whereas the application of radiation in the frequency range 6400-7200 MHz showed no alterations in the heart rate [5]. Thirdly, studies on human [6] and rabbit [7,8,9] erythrocytes have shown that efflux rates of labelled ions through the erythrocyte membranes exposed to microwave radiation (2400 [7] or 2450 [6,9] or 8450 [8] MHz) were identical to control rates, except at critical temperatures where irradiation increased the efflux; thus the effects on erythrocyte permeability are restricted to the narrow temperature range that coincides with the presence of an apparent *membrane phase transition* [9].

Extremely low frequency (50 or 60 Hz) electromagnetic fields generated by transmission power lines and by many convenience appliances,

have also raised considerable concern. Railroad switchyard workers exposed to large sinusoidal 50 Hz electromagnetic fields were found to be subject to elevated chromosome damage and there are other occupational groups exposed *in vivo* to similar electromagnetic fields [10].

Various epidemiological studies have been carried out in order to evaluate the electromagnetic effects on humans, with the most quoted study being that done by Wertheimer and Leeper [11] in Denver, in the late 1970s. It was suggested that a link existed between childhood cancer and an excess of electrical wiring configurations indicating high current flow, and further studies [12] have extended this link to adult cancer.

The implications are that physiological effects may be produced by prolonged exposure to alternating magnetic fields of frequency as low as 60 Hz. These conclusions were reinforced by establishing a relationship between electrical constructions and residences of childhood tumour cases, where the type of construction caused an increase in the magnetic field at the residence [13].

Studies of animal cells and tissues have demonstrated that under certain circumstances weak low-frequency electromagnetic fields can produce changes at the cellular level. Exposure of rat [14] and chick [15] brains to such electromagnetic fields led to the observation of small effects that were strongly dependent on both frequency and amplitude of the radiation. The existence of frequency and power "windows" was corroborated by reports [16,17] that there are ranges of effective frequencies and intensities separated by ranges that are ineffective in causing statistically significant changes in the efflux of calcium ions from chick brains *in vitro* [18,19,20]. For example, at 60 Hz enhanced calcium ion efflux occurred at three intermediate values of field strength, but not at higher or lower values. In addition, when field strength was held constant,

enhanced efflux occurred at a series of specific frequencies, but not at intermediate values. It appears that the combination of frequency and intensity is an important factor determining the extent of changes induced by the field.

Epidemiological and biological studies of animals and whole organs have demonstrated that weak low-frequency electromagnetic fields as well as microwave radiation can produce changes of biological significance. In order to gain a better understanding of the factors influencing the effects of such fields, it is necessary to turn to relevant model systems. Liposomes, consisting only of lipids arranged in concentric bilayers, are recognised as being one of the simplest model systems of a biological membrane, and in fact, they have already been used to consider the effects of applied fields [1,21,22,23]. Many of the effects are subtle requiring particularly well-characterised liposome preparations to ensure unambiguous results, and in general, this condition has not been satisfactorily fulfilled by the previous studies. The purpose of this work has been, accordingly, the preparation and characterisation of liposomes suitable for providing unequivocal interpretation of the effect of applied fields.

According to an informal agreement made at the New York Academy of Sciences meeting on "Liposomes and their Uses in Biology and Medicine", held in 1978, all types of lipid bilayers surrounding an aqueous space are considered to belong to the general category of liposomes [24]. Liposomes can then be classified into three distinct types of vesicles:

i) multilamellar vesicles (MLV) consist of more than a single lipid bilayer and have diameters of 100-5000 nm,

ii) small unilamellar vesicles (SUV) consist of a single lipid bilayer and have diameters less than 50 nm, and

iii) large unilamellar vesicles (LUV) consist of a single lipid bilayer and have diameters larger than 50 nm.

In the early 1960s, it was found that sonication of dispersions of phospholipids resulted in the formation of SUVs [25,26]. Bangham and co-workers [27] were the first to describe the formation of MLVs and since that time the preparation and characterisation of both SUVs, formed using sonication, and MLVs has been extensively researched.

One of the earliest alternatives to sonication as a method of forming liposomes was injection of an ethanolic solution of phospholipid into water [28]. Numerous other techniques for preparing liposomes followed and have been reviewed [29,30,31,32,33].

Generally, unilamellar liposomes are preferred, as studies involving multilamellar liposomes are complicated by the presence of the multiple concentric bilayers and by the difficulty of preparing a uniform size distribution of MLVs.

The surface of SUVs have a high degree of curvature which affects many of the physical properties of their phospholipids such as, for example, molecular packing density and the attendant molecular motions, transbilayer distribution of lipids in multicomponent systems, and thermotropic behaviour of component phospholipids [34]. The outer monolayer of SUVs parallels that of a planar bilayer, but the inner monolayer is subjected to packing constraints unique to systems with a small radius of curvature. Such differences in molecular packing lead to structural asymmetries so that various substances, such as water, might penetrate the inner monolayer of small liposomes less than the corresponding outer monolayer [35]. In addition, because of the strongly curved and elastically strained nature of SUVs, there is a strong tendency for their spontaneous fusion with time [36].

Biological membranes are thought to be better represented by LUVs [37]; packing constraints of the lipids in such liposomes are of no great significance and, in addition, properties such as the large entrapped volume of solution make LUVs the preferred form of liposome to be used as a model system.

Liposome size depends not only on the values of physical and chemical parameters of the system, but also on the method of preparation. LUVs have been prepared by a number of methods, including ultrasound irradiation of MLVs [38], Ca^{2+}-induced fusion (limited to acidic phospholipids) [39], ether infusion [40], reverse phase evaporation [41], French press [42], extrusion [43], and removal of detergent from lipid/detergent micelles by dilution [44], gel filtration [45,46], specific binding to bio-beads [47,48] or dialysis [49,50]. In this work several methods have been used to prepare LUVs and the resulting liposomes carefully characterised since, at least to some extent, conflicting findings on the effects of exposure to magnetic fields may well be due to differences in the characteristics and behaviour of liposomes prepared using different lipids and methods of preparation [22].

In the following section the methods of preparation of the liposomes will be described. Particular attention has been directed to the characterisation as the liposomes formed must be reproducible with every preparation in order to achieve meaningful comparisons of results when fields of various types and magnitude are applied. Effects of applied fields have been linked to changes, in the arrangement of the lipid molecules forming the bilayers, which affect the permeability of the membrane. In this work a solute was trapped in the internal volume of the liposomes and spectroscopic techniques, fluorescence and absorption, used to detect its presence in the external medium after application of a field.

CHAPTER 3 - METHODS OF PREPARATION AND CHARACTERISATION

Details of the lipids used are given in Appendix I.

3.1 *SONICATION*

Sonication induces collisions of the lipid particles, which disintegrate on collision to form many small fragments. These fragments then reaggregate until the resultant bilayer is large enough to seal forming a closed sphere, minimising unfavourable interactions of water with hydrophobic surfaces.

Until recently, sonication had been the most widely used method of preparing SUVs [25,34,38,54]. It is recognised that high-energy sonication, using a probe-type sonicator, will cause oxidation and degradation of the phospholipids [55,56], which is reduced to a certain extent by controlled conditions. Any solute molecules present may be damaged and titanium particles from the probe contaminate the resulting solution of liposomes. Some of the problems can be solved by using a bath-type sonicator with low-energy sonication, but this procedure varies in efficiency usually resulting in a more heterogeneous population, requires long periods of sonication, can only be used for small quantities of solution and is still destructive to the lipids present.

Sonication carried out in this work utilised the probe-type sonicator. Instrumental settings (for example, power output, tuning of the probe and duration), the geometry of the tip of the probe relative to the sample beaker, the volume, concentration, liquid depth and temperature, the depth to which the tip of the probe is immersed, and the atmosphere surrounding the sample all influence the effect of sonication, particularly the amount of chemical degradation caused by the ultrasonic cavitation [56].

Experimental

The buffer solution was saturated with nitrogen (N_2) before and after the lipid was added to it and the solution was kept under N_2 during sonication, in order to reduce the oxygen present and thus, minimise oxidation of the lipid.

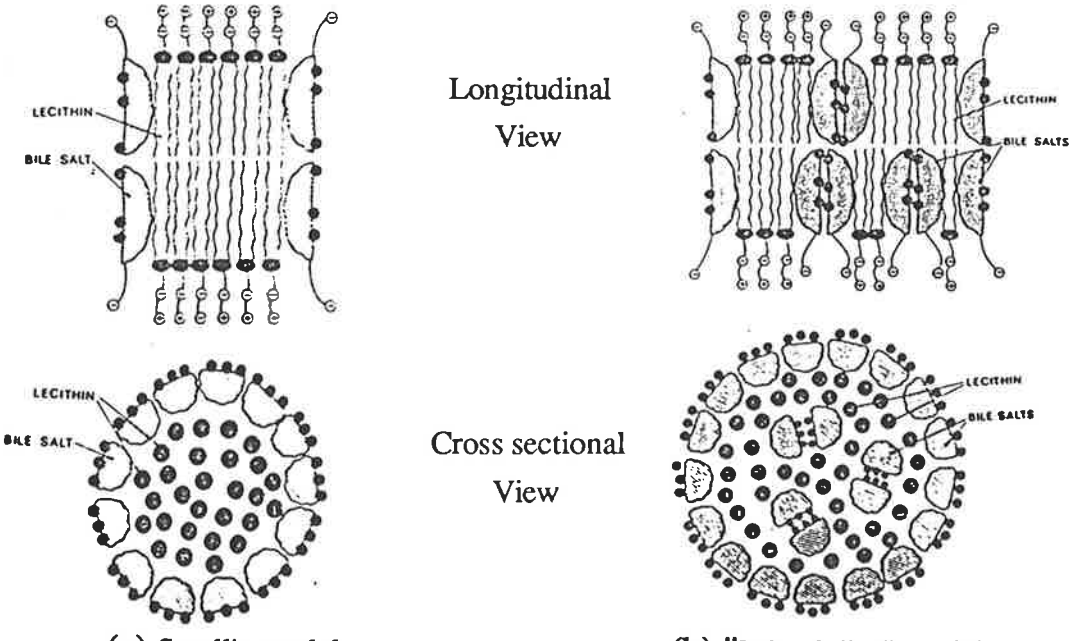
About 10 ml of the buffer containing the lipid (approximately 0.1 $mgcm^{-3}$) was vortexed to obtain MLVs. This phospholipid dispersion was then sonicated using a Branson Sonifier B-12 (Branson Sonic Power Company, Division of Branson Ultrasonics, Co.) at low power (nominal frequency 20 kHz, input power 150 Watts). Sonication was performed in a 25 ml beaker, using the macrotip, at about 65 Watts (corresponding to an output power setting of 3 to 4) for 20 to 30 minutes, or until optical clarity was achieved. The beaker was immersed in an ice-water bath and magnetic stirrers used to stir both the bath and the lipid solution. Intermittent sonication was sometimes necessary to keep the temperature of the solution at an acceptable value.

Following sonication, the lipid solution was centrifuged using a Beckman Model J2-21 Centrifuge, on a setting of (19,000 rpm) for about 30 minutes, at 2° C. This was necessary to remove any titanium particles released from the tip of the probe, as well as any MLVs and undispersed phospholipids.

Passive entrapment of solutes : Passive entrapment of solutes into the interior of liposomes was achieved by adding the desired amount of the solute to the buffer used to hydrate the lipid film. After sonication and centrifugation, the unencapsulated dye was removed from the external buffer by gel chromatography.

3.2 *DETERGENT REMOVAL*

Detergents possess the ability to solubilise lipid bilayers by forming thermodynamically stable macromolecular aggregates known as mixed micelles. Small [57] suggested that a mixed micelle consisted of a lipid bilayer disc surrounded on its perimeter by bile salts (a class of detergent) oriented so that the hydrophilic surfaces face the aqueous solution, while the hydrophobic surfaces interact with the hydrocarbon chains of the lipid (Fig. 3.1 (a)). This model was extended by Mazer, *et al.* [58] who proposed that bile salts exist not only on the perimeter of lipid bilayers but are also incorporated within their interior in high concentrations (Fig. 3.1 (b)). Experimental observations have been explained more fully by the "mixed disc" model, confirming that it is a better model for the structure of mixed micelles of lipid and detergents.



(a) Small's model (b) "Mixed disc" model

FIGURE 3.1 : Schematic models for the structure of the bile salt-lecithin mixed micelle, shown in longitudinal (cut through the disc diameter) and cross section (cut through middle of the hydrocarbon steroid parts and fatty acid chains of bile salts and lecthins, respectively). (a) Small's mixed micellar model [57] (b) "mixed disc" model [58]

Kagawa and Racker [49] were the first to demonstrate that removal of detergent from mixed micellar solutions of lipids and bile salts resulted in the formation of liposomes. Small, *et al.* [59] had already observed that mixtures containing ratios of bile salt to phospholipid of 1:2 in excess water gave "lamellar structures".

As the lipid to detergent molar ratio determines the size of the mixed micelles [60,61], it is expected that it will also affect the size of the liposomes prepared from the mixed micellar solution. The kinetics of detergent removal from mixed micelles is also thought to influence the size of the resulting liposomes [45]. Preparations of liposomes having different diameter sizes are possible by varying the amounts of detergent and lipid added to the aqueous solutions. Clarification of these solutions is assumed to be the endpoint of mixed micelle formation and this state should be reached before commencing detergent removal.

Gel filtration [45,46,62] is a rapid and efficient way of removing the detergent but another technique, involving the more conventional method of dialysis of lipid/detergent micelles [50,63,64,65,66,67] has also been widely used. Special dialysis cells have been designed allowing controlled and reproducible detergent removal at a defined temperature, resulting in the generation of a single, homogeneous population of unilamellar liposomes. The technique is gentle, organic solvents are not used, and the sample is not diluted. The parameters that can influence size can easily be varied, for example, the rate of dialysis (involving the type, thickness, and molecular weight cutoff of the dialysis membrane used), type of detergent, type of lipids, lipid/detergent molar ratio, concentration of lipid, electrolyte content, and pH.

3.2.1 Gel Filtration

Lipid (1-2% (w/v)) was added to the buffer and this solution was vortexed, resulting in a milky dispersion. Sodium cholate was added to obtain a final concentration of at least 0.03 M (ie. 1.3%), which was sufficient to disperse the MLVs. The clear solution of small mixed micelles was applied to a Sephadex G-50 column (fine particle size; 20 × 1.5 cm) to achieve separation of liposomes, which were eluted at the void volume, and the cholate micelles [45]. It has been reported [45] that less than 1% of cholate remained in the preparation, but a second chromatography reduced the residual detergent even further.

Sample volumes and column dimensions were chosen to ensure the complete separation of liposomes and detergent micelles.

As the lipid is known to adsorb to the gel particles [38], all columns were presaturated with the lipid dispersion and then washed and equilibrated with the buffer. After detergent is used on the column part or all of the adsorbed lipid will be removed and the column must be resaturated [68]. If this procedure is carried out lipid recovery after chromotography is high.

Passive entrapment of phenol red : Passive entrapment of solutes into the interior of liposomes was achieved following a technique developed by Allen [69]. The solution containing phospholipid, detergent, and phenol red was loaded onto a column which had been presaturated with an equal volume of detergent-free phenol red. The phospholipid/detergent solution moves faster through the column than the free dye and will overtake the preloaded dye 1 to 2 cm down the column where liposome formation and entrapment of phenol red takes place. Liposomes containing trapped dye

appeared in the void volume, while free dye and detergent eluted at larger volumes.

3.2.2 Dialysis Using the Lipoprep

Initially, the buffer solution, with appropriate amounts of lipid and detergent added, was vortexed and then sonicated using a Branson Sonifier B-12, at about 65 Watts (output power level of 3 to 4) for 15 minutes. It was then necessary to centrifuge the solution for about 15 minutes, using the Beckman Model J2-21 Centrifuge (19500 rpm) before the solution was ready to be dialysed.

Another method of preparing the lipid/detergent solutions was adopted to avoid the problems caused by sonication. The lipid was added to an organic solvent, usually ethanol, methanol or chloroform, in a pear-shaped flask. Use of a rotary evaporator, with water aspirator, for about one hour removed the organic solvent and deposited the lipid as a thin film on the walls of the flask. During this process a water bath was used to keep the temperature above that causing the gel to liquid crystalline phase transition of the highest melting lipid used. The flask was then put in a dessicator under vacuum and left for about 10 hours to remove any remaining solvent.

A portion of detergent was usually added to the organic solvent and deposited as a thin film with the lipid as this increased the rate of solubilisation of the lipid film [70]. The remainder of the required detergent was then added with the buffer at a temperature above the phase transition temperature of all of the lipids used and the lipid film dispersed with gentle shaking. This solution was then left to stand until the solution was clear indicating the complete formation of mixed lipid/detergent micelles. Given an efficient method of removal of the detergent from the homogeneous

mixed micelles the population of resulting liposomes was expected to be relatively monodisperse.

The clarified mixed micellar solutions were added to the Lipoprep® -GD-1 (Diachema Ltd., Rüschlikon, Zürich) and dialysed against the buffer, using high permeability cellulose disc membranes with a molecular weight cutoff (MWC) of 5000 (Type 10.14). The speed with which the apparatus was rotated in the buffer solution was usually 12 rpm and the temperature kept above the phase transition temperature of the lipids used.

Passive entrapment of solutes : Passive entrapment of solutes into the interior of liposomes prepared using the Lipoprep device necessitates following a careful procedure. The small water-soluble compounds should be dissolved in the buffer at the same concentration as that of the mixed micellar solution, otherwise they will be dialysed out. The external buffer solution should contain the solute for, at least, the first few hours of dialysis. Studies [67] have shown that with the lipid/cholate system liposome formation is accomplished after 2 hours and the lipid/n-OG system requires only 1 hour. Once the compound is trapped in the sealed liposome it is no longer required to be in the external solution.

Entrapment is a very tedious procedure when undertaken at the same time as dialysis is performed. It is necessary to place the Lipoprep device in a container capable of holding the 9 dm^3 of buffer solution necessary to ensure removal of the residual detergent. It would be possible to use a smaller container initially but a longer dialysis time would be required. Use of an expensive solute also makes the recovery of the non-entrapped solute desirable, necessitating another involved process.

3.3 *EXTRUSION*

A technique for the rapid production of LUVs by repeated extrusion, under moderate pressures, of MLVs through polycarbonate filters was demonstrated by Hope, *et al.* [43]. This was an extension of the procedure developed by Olson, *et al.* [71] which used extrusion to increase the trapped volume of MLVs. The advantages of this method of preparation are as follows:

i) direct production of unilamellar vesicles by extrusion techniques (LUVETS and SUVETS) from MLVs, formed by simple hydration of dry lipid,

ii) it is a rapid and simple procedure,

iii) all lipids and lipid mixtures which assume bilayer structure on hydration can be used,

iv) lipid concentrations can be varied up to 400 $mgcm^{-3}$,

v) high-trapping efficiencies can be achieved,

vi) no organic solvents or detergents are present,

vii) there is little or no loss of lipid or sample during liposome preparation,

viii) the polycarbonate membranes used in the extruding device do not interact with liposomes containing charged groups, and

ix) homogeneous size distributions of liposomes are obtained with mean diameters from 50 to 400 nm, depending on the pore size of the filter.

It has been reported that repetitive freeze-thaw cycles enhance the trapped volumes, trapping efficiencies, and equilibrium transmembrane distributions of solutes present in the buffer [43,72,73]. During freezing, the transient destabilisation of the bilayer structure aids in the encapsulation of solutes [74] and promotes equilibrium solute distributions across the membrane [73]. Together with the larger diameter this ensures that a higher proportion

of water-soluble compounds can be trapped in the aqueous interior of the liposomes formed using a freeze-thaw procedure followed by extrusion.

Experimental

About 10 cm^3 of a suitable organic solvent, usually n-hexane, was filtered through a Millipore filter (pore size 0.45 μm) several times before being added to the lipid (up to 400 $mgcm^{-3}$) in a pear-shaped flask. This clear solution was then put on a rotary evaporator for two hours and the solvent evaporated off, leaving a thin film of lipid deposited on the walls of the flask. The flask was put in a dessicator under vacuum and left for about 10 hours to remove any remaining solvent. If necessary, the buffer solution was heated to a temperature above that causing a transition from the gel to liquid-crystalline phase of the highest melting lipid. Asolectin has a phase transition temperature below zero (approx. -10° to -15° C) so solutions were prepared at room temperature, but when DPPC is used buffers needed to be heated to about 50° C, well above the phase transition temperature of about 41° C. Vortexing of this solution resulted in the formation of MLVs.

All LUVETs (large unilamellar vesicles by extrusion technique) underwent a freezing and thawing procedure, usually performed 8 to 10 times, before extrusion. The solution of MLVs was frozen using liquid nitrogen and thawed using a warm bath at 40°-60° C. The frozen and thawed MLVs (FATMLVs) were then ready for extrusion utilising the Extruder (Lipex-Biomembranes Inc., Vancouver, B. C., Canada).

The solution of FATMLVs was injected into the chamber above the Nucleopore polycarbonate filters with 100 nm diameter pore size and nitrogen pressure applied via a standard gas cylinder fitted with a high pressure regulator. Pressures of about 1700 kPa (or 250 psi) were used to extrude the vesicles through the filter into the sample tubes. Lipid composition, lipid concentration, buffer composition, and operation

temperature all affect the pressure required for extrusion. The majority of LUVETs were prepared by passing the MLV solutions through two stacked filters ten times.

Preparation of DPPC liposomes required the Extruder to be placed on a hotplate and kept at about 50° C, while the samples of liposomes were kept in a water bath, heated to 50° C, throughout the extruding procedure. When asolectin was the lipid used the operation was carried out at room temperature.

Passive entrapment of solutes : Passive entrapment of solutes into the interior of liposomes was achieved by adding the desired amount of the solute to the buffer used to hydrate the lipid film. After extrusion through the polycarbonate filters, the encapsulated dye was removed from the external buffer by gel chromatography.

3.4 *LIGHT SCATTERING*

Quasielastic laser light scattering (QELS) has been used for many years to measure the average diffusion coefficient and the associated hydrodynamic radius of monodisperse suspensions of small macromolecules [75].

QELS, also referred to as dynamic light scattering or photon correlation spectroscopy, employs digital autocorrelation to analyse fluctuations in scattered light intensity generated by diffusion of particles in solution. It is based on the fact that time-dependent coherence of light scattered by a particle is sensitive to particle diffusion which is dependent on the viscosity of the aqueous medium and the size of the particles. Therefore the measured diffusion coefficient can be used to obtain the average

hydrodynamic radius and hence, the mean diameter of a sample of liposomes.

With advances made in the analysis of QELS data [76,77,78], it is now possible to use photon correlation spectroscopy to determine not only the diameter of phospholipid liposomes but also the degree of polydispersity of the sample [34,75,79,80,81]. A check on the polydispersity of the system helps in interpreting the data as it is easy to obtain misleading results for heterogeneous systems exhibiting bimodal or a more complex size distribution.

Varying the scattering angles is another test of homogeneity. If a liposome preparation is homogeneous, all the liposomes scatter the light in the same way and the calculated diffusion coefficient and hence, the measured diameter will be independent of the scattering angle [82]. If the preparation is inhomogeneous, at smaller scattering angles the larger particles are emphasised resulting in larger diameter values calculated for that sample.

Experimental

Light scattering cells were washed in a detergent solution and protected from dust. Immediately before use they were rinsed thoroughly with millipore-filtered water. To further reduce the dust contribution to noise in the QELS analysis, the solution of liposomes was syringed through a Millipore filter of 0.45 μm nominal pore size and collected directly into the light scattering cell. It was necessary to dilute some liposome preparations as multiple scattering occurs if the concentration of the sample is too high.

The light scattering apparatus consisted of a He-Ne laser (λ_0= 632.8 nm) light source, a temperature-controlled scattering cell-holder and

a Malvern K7027 "LOGLIN" Correlator (incorporating a digital 64-channel correlator, a control microcomputer and a magnetic disc storage unit).

The cylindrical light scattering cell was immersed in an index matching liquid bath, thermostatted at 25° C, and an equilibration time of about 15 minutes was allowed before each measurement. The intensity of scattered light was observed with a photomultiplier tube at angles of 90°, 60°, and 45° to the incident beam. The electrical signal was amplified and fed into the correlator, where the data was analysed by a "two parameter cumulants" fitting method. An "exponential sampling" technique was also used to obtain additional information on the polydispersity of the samples.

Calibration of the light scattering apparatus was performed using a standard monodisperse sample of polystyrene latex spheres, with diameter of about 125 nm.

3.5 *GEL CHROMATOGRAPHY*

Gel exclusion chromatography, or gel filtration, was a method used by Huang [38] in one of the earliest characterisations of a liposome preparation. Gel chromatography is a quick and convenient method of analysis when it is necessary to have a reasonably accurate measure of the average size of the liposomes, but only a semi-quantitative measure of polydispersity [83].

As it has been observed that lipids adsorb to the gel beads [38], presaturation of the column with the lipid minimises any effects of adsorption, thus ensuring high lipid recovery and reproducible elution profiles. Before adding the liposome preparations to the columns, they were centrifuged for several minutes to remove very large liposomes that could clog the column.

A mechanical fraction collector was often used and the elution of sample through the column was carried out at a temperature of 4° C. Turbidity (absorbance at 300 nm) was used as a measure of liposome concentration in each fraction of 1 or 2 cm^3 taken from the column. Fractions were sometimes pooled, concentrated using an apparatus which utilised a vacuum to remove the aqueous solution through a membrane, and rechromatographed.

Sepharose 4B [38,45,46] is suitable for use with liposomes having a small diameter, but Sephacryl can analyse much larger particles, with Sephacryl S-1000 [68,83,84] being suitable for size analysis of liposomes with diameters of up to 250 nm.

Blue Dextran 2000 was eluted through the column and the passage of the dark blue band noted to ensure that it was packed properly and to determine the void volume. Blue Dextran 2000 contains some material of very high molecular weight which is excluded from the column. The leading peaks of the elution profile, which contain these very large molecules indicate the void volume. Theoretically, the void volume of the column is 32-36% of the actual volume of the column and the calibration analysis generally confirmed this estimate.

3.6 *^{31}P NMR SPECTROSCOPY*

^{31}P NMR can be used to monitor the phospholipid phosphorous signal intensity to determine the lamellarity of the phospholipid dispersions [37,43,85,86,87]. Addition of an impermeable paramagnetic or broadening agent to the external medium will decrease the intensity of the initial ^{31}P NMR signal by an amount proportional to the fraction of lipid exposed to the external medium.

The paramagnetic ion, Mn^{2+}, in the form of a 5 mM $MnSO_4$ (or $MnCl_2$) solution has been used to "quench" the ^{31}P NMR signal of phospholipids in the outermost monolayer. Addition of Mn^{2+} to a LUV solution should result in a 50% reduction in the signal intensity. A reduction of more than 50% is expected for SUVs, as the number of phospholipids in the external monolayer is greater than that found in the internal monolayer, due to the highly curved nature of the vesicles. MLVs should give residual signal intensities of greater than 50%.

If the vesicles are leaky to Mn^{2+} the ^{31}P NMR spectrum of the same solution obtained several days later will show a further decrease in the intensity of the signal.

^{31}P NMR spectra were obtained before and after a 5 mM solution of Mn^{2+} was added to the vesicle dispersion.

CHAPTER 4 - CHARACTERISTICS OF LIPOSOME PREPARATIONS

As emphasised earlier, liposomes prepared for the purpose of this study must have the following properties:

i) a narrow size distribution and

ii) a relatively large diameter, so as to be capable of sequestering a sufficiently high number of the probe molecules.

Liposomes prepared by the various methods will, accordingly, be evaluated by the extent to which they satisfy these primary criteria.

4.1 *SONICATION*

Typical examples of chromatographic elution profiles, from Sepharose 4B columns, of liposomes prepared by sonication are shown in Figs. 4.1 and 4.2, where turbidity at 300 nm is indicative of the presence of liposomes; details of the conditions of the preparations are provided in the caption to the figures.

Although the elution profiles do not show the characteristic two distinct peaks usually reported for such preparations [38,88], they correspond to the early elution of relatively large, presumably multilamellar, liposomes in the void volume of the column followed by the passage of the smaller unilamellar liposomes in the form of a broad peak, implying considerable polydispersity in size distribution for both populations of liposomes.

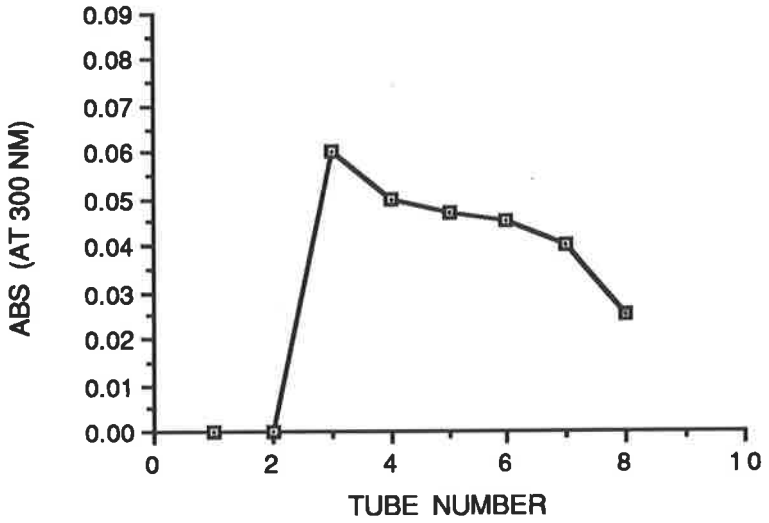


FIGURE 4.1 : Elution profile from a Sepharose 4B column (50 cm×1 cm).
42 mg of asolectin and 2.5 mg of phenol red (approx. 1 mM) were sonicated for 30 minutes in 7 cm^3 of phosphate buffer, pH 7.5, followed by centrifugation for 30 minutes. 0.5 cm^3 of this solution was applied to the column. 5 cm^3 was eluted initially and then 2 cm^3 aliquots were collected in tubes. In tube 9 the faint pink colour of the solution indicated the presence of phenol red (diameter of 5 Å).
Absorbance values were recorded at 300 nm. (The void volume is eluted by tube 5.)

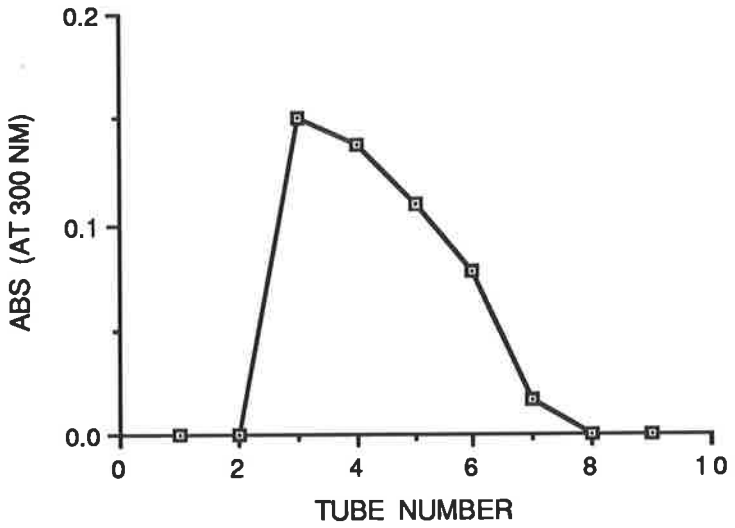


FIGURE 4.2 : Elution profile from a Sepharose 4B column (50 cm×1 cm).
280 mg of asolectin and 2.9 mg of phenol red (approx. 1 mM) were sonicated for 30 minutes in 7 cm^3 of phosphate buffer, pH 7.5, followed by centrifugation for 30 minutes. 0.25 cm^3 of this solution was applied to the column (flow rate : 2 cm^3 per 7.5 mins.). 4 cm^3 was eluted initially and then 2 cm^3 aliquots were collected in tubes. In tube 10 the faint pink colour of the solution indicated the presence of phenol red (diameter of 5 Å).
Absorbance values were recorded at 300 nm. (The void volume is eluted by tube 5.)

Some samples were also investigated by light scattering (Appendix III, Table II), which confirmed the presence of a broad size distribution with a standard deviation of the order of 55% about the mean size. The results obtained reinforce the belief that sonication involves the inhomogeneous disruption of lipid structures [89].

Lack of reproducibility of the physical properties of sonicated samples was understandable considering the difficulty involved in controlling all of the experimental parameters, as discussed in Chapter 3.

It is clear that this particular method of preparation does not fulfill the first requirement of a narrow size distribution of the liposomes. In addition, the amount of phenol red included into the aqueous interior of the liposomes (monitored by measurement of the absorbance at the peak of the spectrum of the neutral dye at 420 nm) was found to be negligibly small. As a result, sonication, as a means of preparing liposomes for the purposes of this work, was considered unsuitable and was not used for further preparations.

4.2 *DETERGENT REMOVAL BY GEL FILTRATION*

Gel chromatography involving the removal of sodium cholate from mixed lipid/detergent micelles was investigated.

Residual cholate levels in the liposomes formed by gel filtration were reported by Brunner, *et al.* [45], to be 2 mole% after Sephadex® G-50 chromatography and by Allen, *et al.* [62], to be 8 mole% after a single passage over the same column. A second passage of the liposomes over

Sephadex® G-50, G-200, Sepharose 4B, or a 12 hour dialysis, reduced detergent levels to below 1 mole% [45,62].

The elution profile from the Sepharose 4B column after using a Sephadex® G-50 column to remove the detergent consisted of a single, relatively symmetrical peak (Fig. 4.3) with almost all the liposomes eluting in the void volume of the column. Slower flow rates would have improved the resolving power of the gel-exclusion columns [68], but to minimise oxidative degradation reasonably fast chromatography is necessary. Homogeneity of the liposome size may be improved by rechromatography on the Sephadex® G-50 column, as shown in Figure 4.4.

In some cases an attempt to include phenol red molecules into the liposomes during preparation was made. Absorption spectra of the eluted liposomes showing little absorbance at 420 nm, the wavelength indicating the presence of phenol red, suggest that very only small amounts were included in the aqueous interior of the liposomes.

One concludes that although the size distribution of liposomes formed by gel filtration may be sufficiently narrow for the purposes of this work, the liposomes will entrap only a small percentage of phenol red in their aqueous interior and thus the resulting preparations would not be suitable. The low trapping efficiency of such liposomes has been noted previously [69], with another disadvantage being the two-fold dilution of a sample each time it passes through a column.

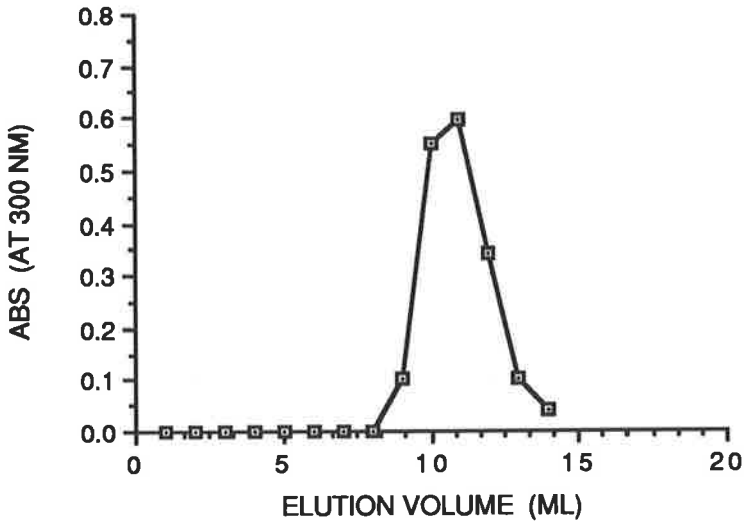


FIGURE 4.3 : Elution profile from a Sepharose 4B column (50 cm×1 cm).
80 mg of asolectin and 1.3% sodium cholate in 5 cm^3 of phosphate buffer, pH 7.5, were applied to a Sephadex ® G-50 column and 1 cm^3 of the eluent was applied to the Sepharose 4B column and the resulting liposomes collected in tubes.
Absorbance values were recorded at 300 nm. (The void volume was about 14 cm^3.)

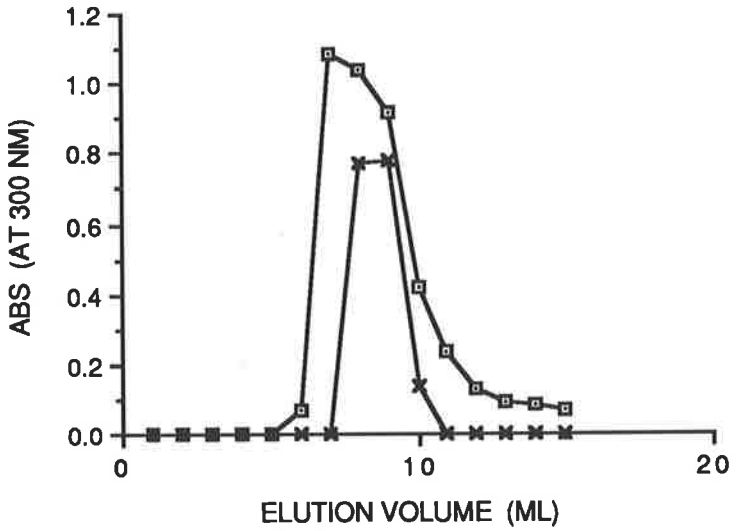


FIGURE 4.4 : Elution profile from a Sephadex ® G-50 column (24 cm×1.5 cm).
56 mg of asolectin, 25 mg of phenol red (ie. 10 mM), and 1.5% sodium cholate in 7 cm^3 of phosphate buffer, pH 7.5, were sonicated for 10 minutes and applied to the Sephadex ® G-50 column. Tubes containing most of the liposomes were concentrated and 1.5 cm^3 reapplied to the column. (□-□-□) First chromatography (×–×–×) Second chromatography
Absorbance values were recorded at 300 nm. (The void volume was about 14 cm^3.)

4.3 *DIALYSIS TO REMOVE DETERGENT*

Controlled dialysis using the Lipoprep device provided an efficient method of removing the detergent from mixed lipid/detergent micelles without diluting the samples.

Extensive light scattering measurements were done on each preparation of liposomes using different initial molar ratios of lipid to detergent. The results are summarised in Appendix III, Tables I-VI giving the mean diameter of the liposomes, together with the total spread of values, and the Q factor [75,90] or normalised variance about the mean, which is taken as a measure of the polydispersity of the system. Ideal scatterers, such as highly homogeneous latex spheres, used in the calibration of light scattering apparatus, gave Q values of about 0.05 [50]. The liposome preparations by this method generally had Q values around this value, thus indicating that they are relatively monodisperse.

In general, dialysing for longer than 10 hours did not significantly alter the diameter size which is consistent with the formation of liposomes occurring after a few hours and further dialysis just necessary to remove the residual detergent.

Data for preparations using sodium cholate as the detergent are presented in Appendix III, Table II. The asolectin/sodium cholate molar ratio varied from about 0.2 to 2 in a pH 7.4 buffer, resulting in liposomes with diameters ranging from 37 nm to 61 nm. This is comparable to egg PC/sodium cholate molar ratios of 0.2 to 1.15 giving diameters ranging from 27.4 nm to 40.3 nm [64]. The diameter of liposomes increased with increasing asolectin/sodium cholate molar ratios in buffers of pH 7.4 (Fig. 5, Appendix III-Table II), which is also in agreement with Shankland's

findings [91] that the radius of egg PC/sodium cholate mixed micelles increases with increasing lipid/detergent ratios.

Results indicate that liposomes prepared by removing sodium cholate using the Lipoprep are sufficiently monodisperse samples for the purposes of this work, but the diameters of the liposomes are relatively small. It is reported [92] that, when using sodium cholate as the detergent, the resulting liposomes have the largest diameters at pH 6, and their size decreases as the pH increases. Establishment of a pH gradient requires liposomes to be formed in acidic buffers, but a lower limit is placed on the pH of cholate dialysis procedures. As the pKa of cholic acid is pH 5.5, at a pH of less than 6 non-micellised cholate will precipitate out of the solution [92,93]. For this reason, and also in an attempt to increase the diameters of liposomes, n-OG (n-octyl-β-glucoside) was used as the detergent. A molecular model for the preparation of liposomes by removal of detergent has been formulated [94] to explain why it is that n-OG produces liposomes with larger diameters than those formed when using sodium cholate.

Liposomes containing low residual detergent result from the relatively fast removal of n-OG from phospholipid/detergent solutions; n-OG has a higher CMC value than sodium cholate (Appendix II). It has been used as the preferred detergent in many studies [64,65,67,69,70,95], since it was first introduced [64]. Its very high rate of dialysis and a residual detergent concentration of less than 0.1% in the final liposome preparation compare favourably to the preparation using cholate with 0.5% to 1.5% residual detergent [64,70]. Also clear mixed micellar solutions were obtained more rapidly when n-OG was used.

Data for preparations from phospholipid/n-OG in a phosphate buffer, pH 7.4, are given in Table III. The asolectin/n-OG molar ratio was varied from 0.125 to 0.875 resulting in liposomes with diameters ranging

from 136 nm to 50 nm. Contrary to the trend observed when using sodium cholate in a neutral buffer, liposomes have diameters which increase with decreasing asolectin/n-OG molar ratio (Fig. 4.5, Appendix III, Table III).

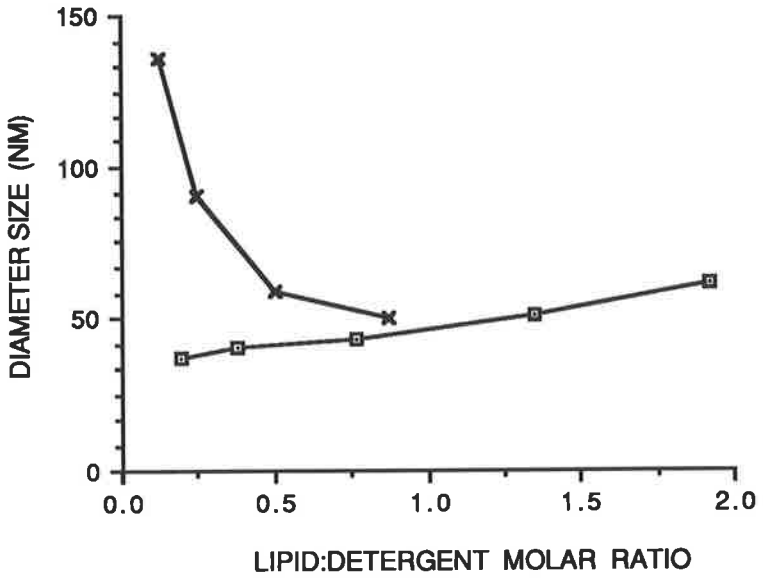


FIGURE 4.5 : Diameter of liposomes against molar lipid/detergent ratio for liposomes prepared using the Lipoprep device. FIGURE 4.5 : Diameter of liposomes against molar lipid/detergent ratio for liposomes prepared using the Lipoprep device.
(x–x–x) Liposomes prepared with asolectin and 1.4% sodium cholate in phosphate buffer, pH 7.4, after 10 hours dialysis. (□-□-□) Liposomes prepared with asolectin and 50 mM n-octylglucoside in phosphate buffer, pH 7.4, with 10 hours dialysis.

Using n-OG as the detergent it was possible to form liposomes in acidic buffers and the data on asolectin liposomes prepared in buffers of varying pH are summarised in (Appendix III, Tables III and IV). All preparations were comparatively monodisperse, but it was difficult to ascertain any trend in the conditions which affected the diameter of the resulting liposomes.

Several preparations resulted in liposomes having diameters around 100 nm, but it was noted that asolectin/n-OG molar ratios below 0.1

in acidic buffers formed relatively monodisperse populations of liposomes with diameters larger than 200 nm after slightly longer dialysis.

Dipalmitoylphosphatidylcholine (DPPC) was used in some of the preparations with DPPC/n-OG molar ratios varying from 0.15 to 0.37, in buffers at pH 3 (Appendix III, Table V). Relatively polydisperse populations of liposomes resulted, with diameters ranging from about 115 nm to 260 nm, with most liposomes having diameters greater than 150 nm.

Another class of non-ionic detergents, the N-D-gluco-N-methylalkanamides (OMEGA, MEGA-9, MEGA-10) are chemical analogues of n-OG and, as they also possess high CMC values (Appendix II), are rapidly removed from solutions by dialysis [96]. It is reported that they have low solubility at low temperatures [97], which could explain why after one week of standing at room temperature a solution of MEGA-10 with asolectin in an acidic buffer was still not clear. Attempts to form mixed micelles using the zwitterionic detergent, CHAPS, in an acidic buffer also failed to produce a clear solution of mixed micelles after standing for one week. A reasonably clear solution of asolectin/MEGA-9 mixed micelles was formed after a normal standing time (of about 1 hour). Asolectin/MEGA-9 molar ratios varying from 0.15 to 0.27 in buffer solution, pH 3.6, resulted in relatively polydisperse populations of liposomes, with diameters ranging from about 50 nm to 105 nm (Appendix III, Table VI).

Several of the dialysed preparations had their diameter sizes redetermined using light scattering some time after their formation and initial characterisation. Up to 10 days after formation the diameter of the liposomes had changed by less than 10%. As the diameters usually decreased or increased only slightly it was evident that the liposomes were sufficiently large so as not to fuse together, a problem often encountered when storing

SUVs for longer periods. Polydispersity usually increased slightly after standing for several days.

For liposomes prepared from the same lipid/detergent molar ratio, but on different days, the agreement between the measured diameters was usually within ±(10 to 15) nm.

In summary, liposomes formed from asolectin in buffers of varying pH and using either n-OG or MEGA-9 as the detergent have very narrow size distributions and also have sufficiently large diameters for inclusion of probes.

Although controlled dialysis using the Lipoprep ensures that residual detergent in the preparations is kept to a minimum, the retention of even a small amount of detergent is unavoidable. Any detergent present in the bilayer structure is likely to increase the passive permeability and decrease the stability of the membrane. Given this, it was considered necessary to consider another form of preparation.

4.4 *EXTRUSION*

The extrusion technique offers an alternative method of preparation which does not involve the use of solvents or detergents and which produces liposomes with a high trapping efficiency.

Populations of LUVs prepared with 2-10 $mgcm^{-3}$ of asolectin in buffers at pH 3.6 to 7.3 were relatively monodisperse with diameters ranging from 88 nm to 140 nm, depending upon the buffers used (Appendix III, Table VII). Up to 13 days after preparation, light scattering measurements showed a less than 2% change (either increase or decrease) in

the diameter size compared to measurements done when the liposomes were initially formed.

Studies performed on liposomes prepared by extrusion produced typical spectra with "bilayer" line shapes having a high-field peak and a low-field shoulder. Addition of a paramagnetic cation, Mn^{2+}, broadened the peak as it quenched the signal of phospholipids that it could approach. A reduction of approximately 50% of the signal indicated that the majority of liposomes produced by extrusion were indeed large and unilamellar.

Liposomes prepared from DPPC in a pH 7.3 buffer were more polydisperse and generally had larger diameters ranging from 130 nm to 174 nm.

Results indicate that extrusion of certain lipid solutions results in monodisperse populations of LUVs having diameters around 100 nm. Therefore, these liposomes satisfy the criteria for this study, and have the added advantage of being free of membrane-perturbing contaminants.

CHAPTER 5 - STUDIES ON FIELD EFFECTS

As stated earlier, biological membranes are suggested as the possible site of interaction between non-ionising radiation and biological systems. It is important to determine whether proteins, phospholipids, or both are participating in any membrane response to fields by examining effects on the lipid bilayer in the absence of membrane proteins, on protein components alone, and on combinations of the two.

The interaction mechanism, although still not known, appears to involve a change in the permeability of the membranes after exposure to fields [23]. Research previously undertaken, using both biological membranes and model systems, suggests that fields interact with membranes in such a way as to create pores rather than causing complete breakdown and degradation of the bilayers [23,98].

As an extreme case, exposure of liposomes to electric fields exceeding 30 $kVcm^{-1}$ has resulted in enhanced permeability of trapped ions [99,100]. The leakage is attributed to the field-induced transmembrane potential (in this case, about 200 mV), which allows the passage of ions through the membrane. This electrical breakdown of the membrane is completely reversible without any adverse side effects, as long as the exposure time is not too long or the field intensity is not too high [98].

Microwave fields have also affected the diffusion of ions through biological membranes [9]. As in the case of exposure to high electric fields, this enhanced permeability is reversible and transient, so that immediately following termination of the exposure the diffusion is significantly reduced and eventually (after about 1 hour) returns to normal. Results found by Liburdy and Magin [23] also demonstrate that phospholipid liposomes are

influenced by non-ionising radiation, as they become permeable and release entrapped drugs when irradiated by microwaves.

This work aims to further investigate the effects of fields on liposomes consisting only of phospholipids, as this is the first step in understanding the responses of biological membranes to irradiation.

5.1 *PROBES*

Radioactive tracer methods have been widely used to monitor transport of ions across membranes but absorbance and fluorescence measurements of suitable probes are very sensitive alternative methods. In this work spectroscopic methods were used to investigate the release of a number of probes.

5.1.1 Phenol Red [101]

Phenol red, a pH-sensitive dye, is incorporated into liposomes prepared in a buffer solution, at pH 7. After removal of the unencapsulated dye from the external medium the suspension of phenol red-containing liposomes should be a cloudy yellow-orange colour. After application of a field the pH of the external medium is increased from 7 to 9.4 by adding NaOH and any dye which has been released undergoes a distinct colour change to red-purple. The acidic and alkaline forms of phenol red have absorption maxima at 420 nm and 520 nm, respectively.

As the NaOH is added after the field has been removed there is no pH gradient across the membrane during its application. This ensures that changes observed are associated with dye leakage and not with membrane permeability to H^+ and OH^-.

Experimental

Phenol red was added to the 5 mM potassium phosphate, 150 mM KCl buffer (pH 7.4) either before or at the same time as the buffer was added to the lipid. Solutions of phenol red (0.4 $mgcm^{-3}$/1 mM or 3.5 $mgcm^{-3}$/10 mM) were prepared with the lipid concentration varying from 6 to 40 $mgcm^{-3}$. Sonication was carried out as described earlier and the resulting cloudy yellow-orange coloured solution was applied to a Sephadex G-50 (fine particles; 20 cm × 1.5 cm) column using the phosphate buffer (pH 7.4) as the eluent. The unencapsulated dye was separated from the phenol red-containing liposomes eluting in the void volume.

Solutions of 3.5 $mgcm^{-3}$ (10 mM) or 18 $mgcm^{-3}$ (50 mM) phenol red were also prepared in a 20% sodium cholate solution with 10 $mgcm^{-3}$ of asolectin. Gel filtration was performed on a Sephadex G-50 (fine particles; 20 cm × 1.5 cm) column to remove the detergent and form liposomes containing phenol red.

The solution eluted by the columns was collected in 1 or 2 cm^3 samples by utilising the fraction collector. A cloudy solution indicated the presence of liposomes. Absorption spectra of those samples containing liposomes were recorded at room temperature, using a Varian Cary 219 spectrophotometer.

5.1.2 Tris (2,2'-bipyridyl) ruthenium (II) ion [$Ru(bipy)_3^{2+}$]

This system uses the fact that $Fe(CN)_6^{4-}$ quenches the fluorescence of $Ru(bipy)_3^{2+}$. Liposomes are prepared with $Ru(bipy)_3^{2+}$ present in the buffer. During formation it is included into the liposomes and the unencapsulated complex ions removed by use of gel chromatography. $Ru(bipy)_3^{2+}$ has an emission maximum at about 650 nm with an excitation

wavelength of 453 nm, when liposomes are present. When $Fe(CN)_6^{4-}$ is added to the solution of liposomes, it is expected that there will be very little, if any, quenching of the fluorescence signal because of the absence of $Ru(bipy)_3^{2+}$ in the external buffer.

A field can then be applied to this system and any perturbation allowing $Ru(bipy)_3^{2+}$ to be released from the interior of the liposomes will be reflected in a decrease in the fluorescence intensity when the complex ions become exposed to the $Fe(CN)_6^{4-}$.

Total disruption of the liposomes would lead to the signal being fully quenched. It would be distinguishable from the formation of pores as complete release of $Ru(bipy)_3^{2+}$ into the external buffer via pores is unlikely and would require a considerable amount of time.

Experimental

Tris (2,2'-bipyridyl) ruthenium (II) chloride hexahydrate [$Ru(bipy)_3Cl_2.6H_2O$] was added to the 20 mM Hepes buffer (pH 7.3) and this solution used to dissolve the lipid film. Solutions of $Ru(bipy)_3 Cl_2.6H_2O$ (0.5 $mgcm^{-3}$/0.7 mM) were prepared with a lipid concentration of 10 $mgcm^{-3}$. The solution was vortexed before carrying out the freeze-thaw procedure followed by extrusion using the Rapid Extruder. The resulting yellow-orange coloured solution was applied to a Sephadex G-25 (fine particles; 20 cm × 1.5 cm) column using the Hepes buffer (pH 7.3) as the eluent. Liposomes containing $Ru(bipy)_3^{2+}$ eluted in the void volume, while the much smaller free ions were retarded by the gel and were eluted several tubes later. Utilisation of the fraction collector enabled collection of 1 or 2 cm^3 samples from the column. The tubes with a distinctive yellow-orange colour and also opalescence due to the presence of liposomes were used in further studies. Various amounts of $K_4Fe(CN)_6$ were added to the cuvettes,

which were inverted, with parafilm covering the opening, to mix the contents.

Fluorescence measurements were recorded on a Aminco SPF-500 spectrofluorimeter, with excitation at 453 nm and observation of the emission maximum at about 650 nm. The cuvette and its contents were kept at a constant temperature by a circulating water bath.

5.1.3 Fluorescent Amines

Studies [102,103,104] have shown that lipophilic cations and drugs can be accumulated efficiently into liposomal sysyems in response to establishing a K^+ diffusion potential (inside negative) across the membrane. Alternatively, some drugs can be loaded into the interior aqueous space for *in vivo* drug delivery when the liposomes have a transmembrane pH gradient (interior acidic) [105]. Trapping efficiencies, drug retention times, and concentrations of entrapped drugs achieved in this way are superior to those possible by passive trapping procedures.

In the presence of a pH gradient, fluorescent amines move into the internal volume of liposomes where their fluorescence intensity may be quenched [106]. This behaviour allows the ΔpH across membranes to be measured. Some fluorescent amines, such as acridine hydrochloride and quinine, show a pH-dependent shift of their emission maximum [107], which is also useful in determining the internal pH of liposomes. This method has been utilised in this work as it involves the use of a much lower probe concentration than that required by the fluorescence-quenching method, and therefore should have a less perturbing effect on the natural responses of the membranes.

When added to a solution of acidic liposomes in a more alkaline medium, the amine molecule is expected to accumulate in the internal

aqueous space in its neutral form and become charged as it accepts a proton from the acidic medium. Any pores created during application of a field would allow the protonated trapped amine molecules into the excess external buffer. Thus, any response to the field can be monitored by observing the fluorescence emission spectrum of the amine. If the field perturbs the membrane it is expected that there will be a shift of the emission peak indicating that the amine has moved from an acidic environment to a more alkaline one.

Experimental

The fluorescent amines chosen as having the desired properties were acridine hydrochloride and quinine. Liposomes were prepared in the usual way using sonication, detergent removal employing the Lipoprep device, and extrusion through the Rapid Extruder. The buffer used to dissolve the lipid was 20 mM glutamate buffer with pH ranging from 2.5 to 3.5. The resulting acidic liposomes were added to an excess (about 2 cm^3) of 20 mM sodium citrate buffer, pH 6, in the cuvette. A small aliquot of acridine hydrochloride or quinine solution was added and the contents of the cuvette mixed by covering the opening with Parafilm and gently inverting the cuvette.

Fluorescence spectra were recorded with a Aminco SPF-500 spectrofluorimeter, using a circulating water bath to keep the temperature of the solutions in the cuvette constant.

Amine Concentration : To obtain meaningful spectra, the amount of amine present must give a reasonably intense fluorescence peak, but also be such that all of it becomes protonated and is thus sequestered in the acidic interior of the liposomes.

For each system studied, the following calculations were done to determine the concentration of amine needed in the cuvette.

The following notation is introduced:

N moleculesdm^{-3}	the number density of phospholipids
r dm	the radius of a liposome
R dm	the thickness of the bilayer
A dm^2	the cross sectional area of a lipid molecule

It is assumed that the liposomes are large and unilamellar so that the number of phospholipids in the outer and inner layers are equal. The number density of the liposomes, N_ℓ , is

$$N_\ell = N/(8\pi\, r^2/A) \;\; dm^{-3}$$

and the fraction of the total volume entrapped by the liposomes, f_ℓ , is just N_ℓ multiplied by the internal volume of a single liposome,

$$f_\ell = N_\ell(4\pi/3)(r-R)^3.$$

Neglecting the concentration of the hydrogen ions in the outer solution (pH 7) relative to that in the entrapped volume, $(pH)_\ell$, and given that the total volume of the solution is V, the number of moles of entrapped protons, $(n_{H+})_\ell$, is

$$(n_{H+})_\ell = V\, f_\ell\, 10^{-(pH)_\ell}$$

and this is also the number of moles of amine molecules which will become protonated and trapped in the interior of the liposomes.

Generally, a slightly lower concentration of amine than that calculated was added to each cuvette to ensure that all of it could be trapped in the acidic interior of the liposomes.

The following assumptions were made: i) the liposomes are LUVs; ii) R, bilayer thickness is about 50 Å; iii) the cross-sectional area of a phospholipid molecule is 60 $Å^2$; iv) accurate determinations of the diameter of liposomes were made in light scattering measurements and v) the concentration of phospholipid in solution is equal to that of the phospholipid dispersion of MLVs obtained prior to formation of LUVs.

5.1.4 5,6-Carboxyfluorescein

5,6-CF is one of the most commonly used fluorescent markers to assess the rates of leakage of water-soluble substances from liposomes. Since the introduction of this simple fluorescent method in 1977 [108], it has been used in measuring liposome-cell interactions [109,110,111,112] and liposome stability [111,113,114].

In dilute solutions of 5,6-CF fluorescence is proportional to the number of dye molecules present, but as the concentration increases above 10 mM the yield per molecule decreases because of the interaction between the fluorophore molecules. The fluorescence of 5,6-CF is quenched at high dye concentrations inside the liposomes (about 95% quenching for concentrations over 100 mM [111]), but increases when the dye is released and diluted into the external medium. When a field is applied, any perturbation of the membrane that would cause release of 5,6-CF molecules may be monitored by an increase of the fluorescence intensity of the emission peak at 520 nm (with excitation wavelength set at 490 nm).

5,6-CF is considered to be a better choice of probe than its parent compound, fluorescein, as it is more polar and hence leaks out of the liposomes more slowly [108]. Therefore the natural leakage of the dye from the liposomes is less likely to be interpreted as an effect of the application of a field.

Experimental

A solution of asolectin (11 $mgcm^{-3}$), 5,6-CF (37 $mgcm^{-3}$) and sodium cholate (1.3%) was vortexed and then applied to a Sephadex G-50 column (fine particles; 20 cm × 1.5 cm) in order to form liposomes containing 5,6-CF by gel filtration. The column was equilibrated with lipid in the 5 mM potassium phosphate, 150 mM KCl solution, at pH 7.5, and this buffer was also used as the eluent. A second chromatograph of this solution further separated the liposomes containing 5,6-CF from the free dye and also removed any residual detergent.

Extrusion was also used as a method of preparation of liposomes containing 5,6-CF. Asolectin (10 $mgcm^{-3}$/12.5 mM) and 5,6-CF (7.5 $mgcm^{-3}$/20 mM) were dissolved in a neutral buffer (20 mM Hepes solution, pH 7.5), and extruded through polycarbonate filters using the Rapid Extruder and utilising the freeze-thaw procedure.

A Sephadex G-50 (fine particles; 20 cm × 1.5 cm) column was used as described above to obtain liposomes containing 5,6-CF and no dye in the external medium.

Fluorescence spectra were recorded on a Aminco SPF-500 spectrofluorimeter, with excitation at 492 nm and observing the emission maximum at about 520 nm. The cuvette and its contents were kept at a constant temperature by a circulating water bath. A thermocouple probe has been used to monitor the temperature.

5.2 *FACTORS OF RELEVANCE*

Investigation of the effect of fields on model systems for biological membranes involves the consideration of the following factors.

Disruption of the Liposomes : Triton X-100, a non-ionic detergent, is used in this work to disrupt the membranes and release all of the sequestered solute into the external medium. It is one of the most effective solubilisers of both biological and artificial membranes and is used extensively in reconstitution studies.

When small amounts of detergent are added to liposomes some of it is incorporated into the bilayers without disrupting them. At this stage the permeability of the liposomes may be affected allowing the release of some solute from the interior. As the amount of detergent is increased the bilayers become saturated and additional detergent induces the formation of mixed detergent/phospholipid micelles [115]. Thus there is a special region, where the barrier efficiency of the phospholipid membrane is reduced, which occurs at a far lower concentration than that at which the liposome is destroyed [116]. Treatment of liposomes with detergent is assumed to free all of the solute which then partitions into the aqueous phase. Reports [112,117,118,119,120,121] have indicated that the amount and rate of release of solute upon addition of detergent is a function of the type of liposome (that is,whether they are MLV, SUV, or LUV), lipid composition of the liposome, Triton concentration, and temperature and duration of detergent incubation.

In general, a solution of Triton X-100 at a low concentration was added to the liposome suspension, the cuvette shaken and sufficient time allowed to ensure complete disruption of the liposomes.

Quenching of the Fluorescence by Other Ions : The presence of some ions can affect the fluorescence observed. For example, quinine is extremely sensitive to quenching by chloride ions [107], necessitating that the pH adjustment of buffers was carried out using H_2SO_4 and NaOH.

Entrapment of Solutes : Efficiency of entrapment of a solute into liposomes is dependent upon several factors [122], such as type of liposomes, lipid composition of liposomes, charge of liposome surfaces, aqueous buffer strength, and nature of the compound to be entrapped. The solute used should i) be water-soluble with a low affinity for the lipid bilayer; ii) be stable during liposome formation; and iii) not react with the polar head groups of the phospholipids.

Column chromatography, which is necessary to separate liposomes from non-entrapped solutes, dilutes the sample necessitating a concentration procedure which utilises a vacuum technique to remove some of the solvent.

Osmotic Gradient : The osmotic gradient across the phospholipid membrane, which is created with the inclusion of solutes, should be relatively small and not be a perturbing influence on the system, as the unilamellar liposomes used have diameters between 30 nm and 200 nm. It is therefore not necessary to include permeating neutral ions, such as glucose or urea, which would translocate across the liposome and dissipate any osmotic gradient [123].

Background Fluorescence : The buffers used throughout showed no fluorescence except a small peak at about 385 nm, due to the Raman scattering of water.

A high degree of Rayleigh scatter is associated with suspensions of large liposomes [35]. To evaluate the extent of this extraneous scatter, fluorescence measurements involved obtaining spectra for the buffer, the buffer with the liposome suspension added, and finally, the spectra of the amine after it was added to the solution of buffer and liposomes. All spectra have had the emission from the buffer and liposomes subtracted. This

background fluorescence was kept as low as possible, so as not to interfere with the fluorescence due to the probe.

Temperature : It is well-established that the liposomal permeability to aqueous solutes is greatly increased at the thermotropic (gel to liquid crystalline) phase transition temperature of the relevant lipids used [124,125,126,127,128]. At this temperature the bilayer changes from a crystalline solid (gel) phase, with conformationally ordered hydrocarbon chains, to a liquid (liquid crystalline) phase, with conformationally disordered hydrocarbon chains. Temperature must be carefully controlled and monitored during exposure to fields so that any effect observed can be attributed to the imposition of a field and not just unequal solvent heating.

The pH of a buffer solution is also known to decrease with an increase in temperature [101].

Proton Permeability : Proton permeability is dependent on the buffer used [129]. In the presence of sodium acetate there is prompt equilibration of H^+ concentration across liposomes, but substantial pH gradients are maintained with the other buffers, such as phosphate. As liposome membranes are relatively permeable to chloride [130,131], it is possible that any chloride in the medium could move with a proton and discharge the pH gradient.

Apparent proton transfer rates are substantially greater for single than for multicompartment liposomes.

5.3 *RESULTS*

Spectroscopic methods of following the release of a solute from the interior space of liposomes were investigated using different probes. Each probe was assessed as to whether it would be useful in observing any change in permeability of the lipid membranes after application of a field.

5.3.1 Phenol Red

Phenol red was not a useful probe for ascertaining the extent of inclusion of a water-soluble compound into the aqueous interior of the liposomes. As mentioned earlier, insufficient amounts of phenol red were included into liposomes, thus further investigations using phenol red were not undertaken.

5.3.2 $Ru(bipy)_3^{2+}$

Fluorescence measurements on liposomes containing $Ru(bipy)_3^{2+}$ in a buffer at pH 7.3 resulted in an emission maximum at 650 nm (excitation wavelength set at 453 nm) [Fig. 5.1 i)]. Addition of the quenching ion, $[Fe(CN)_6]^{4-}$, gave a peak of the same shape with only a very slight decrease in intensity, indicating that column chromatography was successful in removing the $Ru(bipy)_3^{2+}$ from the external buffer. After addition of the Triton X-100, there was a considerable decrease in intensity (dependent on the amount of quenching ion present) and the emission peak also underwent a hypsochromic shift to about 635 nm [Fig. 5.1 ii)]. Addition of more quencher caused no shape change but lowered the intensity of the peak [Fig. 5.1 iii)] and confirmed that the decrease of fluorescence intensity was not only the result of adding Triton X-100 but, in fact, a quenching effect from exposure of more $Ru(bipy)_3^{2+}$ molecules to $Fe(CN)_6^{4-}$. As a comparison, the fluorescence spectrum of free $Ru(bipy)_3^{2+}$ in the buffer at pH 7.3 was obtained [Fig. 5.1 iv)]. Presence of liposomes did not appear to affect the

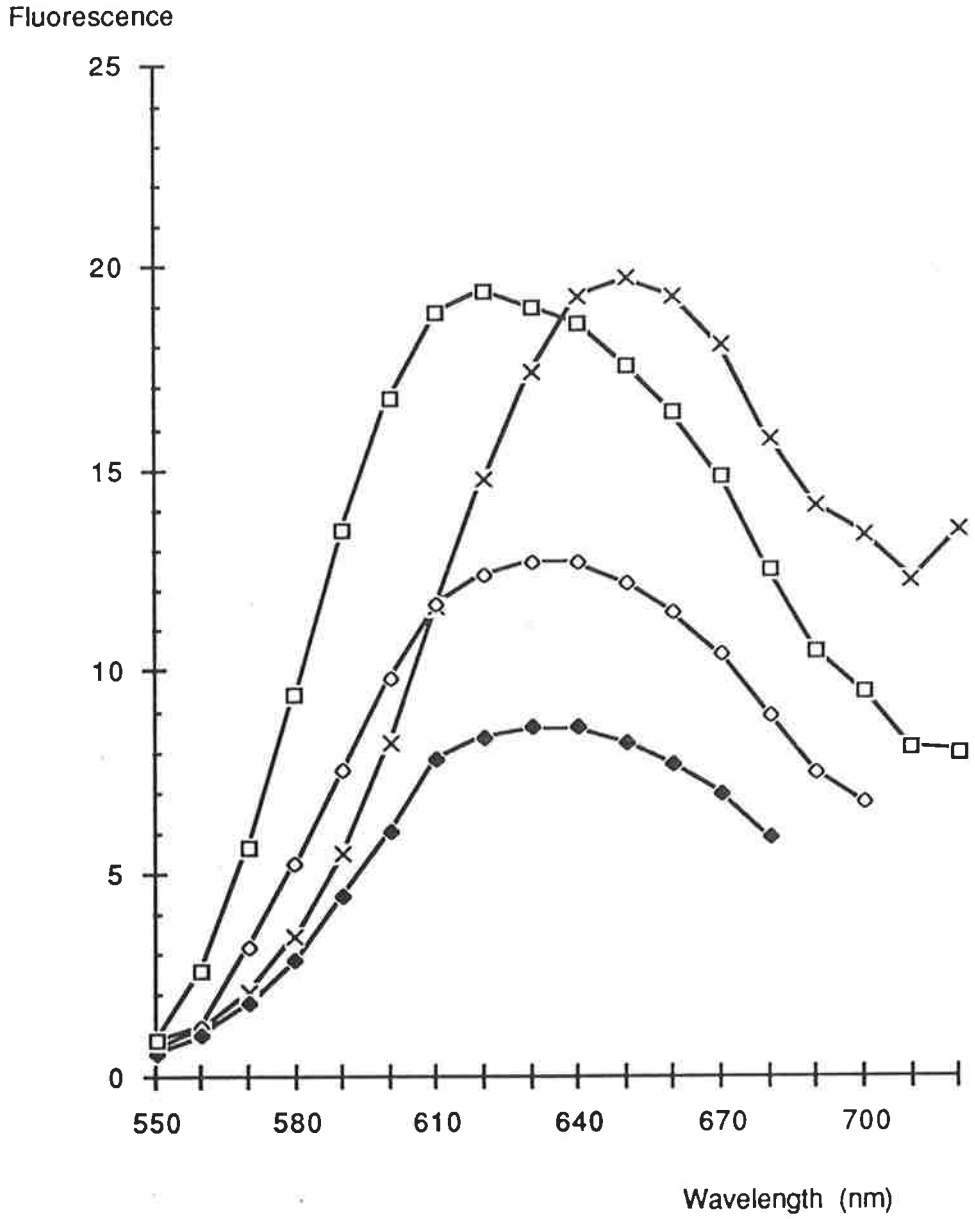


FIGURE 5.1 : Fluorescence intensity against wavelength for the $Ru(bipy)_3^{2+}$ quenching system. Liposomes prepared from 10 mg cm^{-3} asolectin and 0.7 mM $Ru(bipy)_3^{2+}$ in 20 mM Hepes buffer, pH 7.3, and extruded through 100 nm pore size polycarbonate filters.

(i) (×-×-×) liposomes containing $Ru(bipy)_3^{2+}$ just after being passed through the column to remove the free complex ions (ii) (o-o-o) after addition of Triton X-100

(iii) (♦-♦-♦) after addition of more quencher, $Fe(CN)_6^{4-}$, to the solution containing Triton X-100 (iv) (□-□-□) free $Ru(bipy)_3^{2+}$ in 20 mM Hepes buffer, pH 7.3

intensity but did cause a bathochromic shift of the emission peak suggesting that $Ru(bipy)_3^{2+}$ was associated with the liposome surface. The fluorescence intensity of free $Ru(bipy)_3^{2+}$ in buffer was stable for at least 20 hours.

A spectrum was obtained on a preparation immediately after it had been eluted through a column and consisted therefore of only liposomes containing $Ru(bipy)_3^{2+}$ and no ion present in the external buffer [Fig. 5.2 i)]. This solution was then left for four days before the quencher, $Fe(CN)_6^{4-}$, was added [Fig. 5.2 ii)], resulting in a more noticeable (approximately 20%) decrease in intensity due to the release of $Ru(bipy)_3^{2+}$ into the external buffer. Addition of Triton X-100 and addition of more quencher caused the expected hypsochromic shift and a large decrease in intensity [Fig. 5.2 iii) and iv)]. In this case, the emission peak was shifted to 620 nm, which is the same position as free $Ru(bipy)_3^{2+}$ in buffer at pH 7.3. It appears that the older and hence less stable preparations of liposomes are more completely disrupted and the complex ion is then likely to be totally free in the solution. Sometimes liposomes are not totally disrupted allowing some association, which shifts the peak from that of free dye, but usually enough detergent is added to increase solute permeability and decrease the intensity of the peak.

Standing of the liposomal solutions for up to 1 hour generally showed little release of $Ru(bipy)_3^{2+}$ but often after longer periods the fluorescence intensity of the emission peak had increased. This suggests that there may be another quenching mechanism which may become ineffective with time. The slight increase of fluorescence intensity is seen to occur only in solutions containing liposomes, therefore $Ru(bipy)_3^{2+}$ may initially be penetrating into the hydrophobic bilayer structure where its fluorescence is quenched but later increases as the ion again has contact with an aqueous environment.

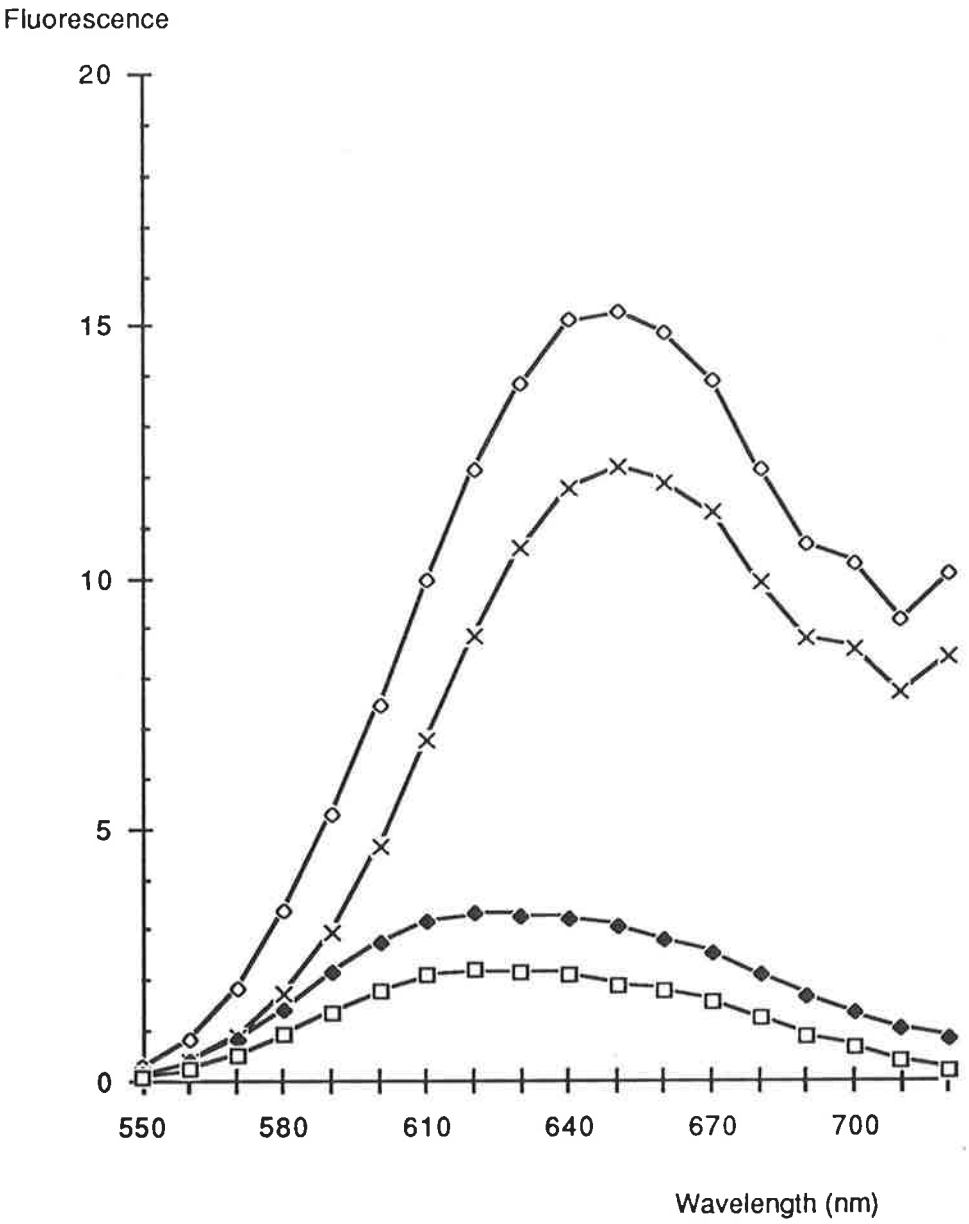


FIGURE 5.2 : Fluorescence intensity against wavelength for the $Ru(bipy)_3^{2+}$ quenching system. Liposomes prepared from 10 mg cm^{-3} asolectin and 0.7 mM $Ru(bipy)_3^{2+}$ in 20 mM Hepes buffer, pH 7.3, and extruded through 100 nm pore size polycarbonate filters. (i) (o-o-o) liposomes containing $Ru(bipy)_3^{2+}$ just after being passed through the column to remove the free complex ions (ii) (×-×-×) after leaving the liposomes containing $Ru(bipy)_3^{2+}$ for 4 days and then adding quencher, $Fe(CN)_6^{4-}$, to the solution (iii) (♦-♦-♦) after addition of Triton X-100 (iv) (□-□-□) after addition of more quencher, $Fe(CN)_6^{4-}$, to the solution already containing Triton X-100

Preliminary experiments involving exposure of liposomes containing $Ru(bipy)_3^{2+}$ to microwave fields were inconclusive. Exposure of these liposomes, with quencher present in the external buffer, to fields of 73 μW (at a distance of 40 cm) caused little change to the fluorescence intensity. Spectra of test sample and control solutions were almost identical up to 24 hours after exposure. After this time a second exposure to the field caused a larger decrease in the intensity of the emission peak of the exposed sample relative to that which occurred for the control. Both the sample and the control showed an increase in fluorescence intensity after solutions were left standing overnight and the position of the peaks indicated some association of $Ru(bipy)_3^{2+}$ to the liposomes, which complicates interpretation of the results. Further studies are required to determine the locations of the fluorescent probe and the quencher in the system and the effects of lipids, quencher, detergent, temperature, and other parameters on the fluorescence intensity of $Ru(bipy)_3^{2+}$. Any intrinsic quenching or enhancement of the intensity must be fully understood, before any meaningful conclusions can be made from the effects of fields on this system. As association of $Ru(bipy)_3^{2+}$ to the phospholipids in the bilayer also occurs this probe was considered unsuitable for further field effect studies.

5.3.3 Acridine Hydrochloride

The effect of pH on the fluorescence emission spectrum of acridine hydrochloride is shown in Fig. 5.3. When the excitation wavelength is set at 350 nm, an acidic peak occurs at 475 nm and an alkaline peak at 425 nm, while a peak at 450 nm is dominant at about pH 6.

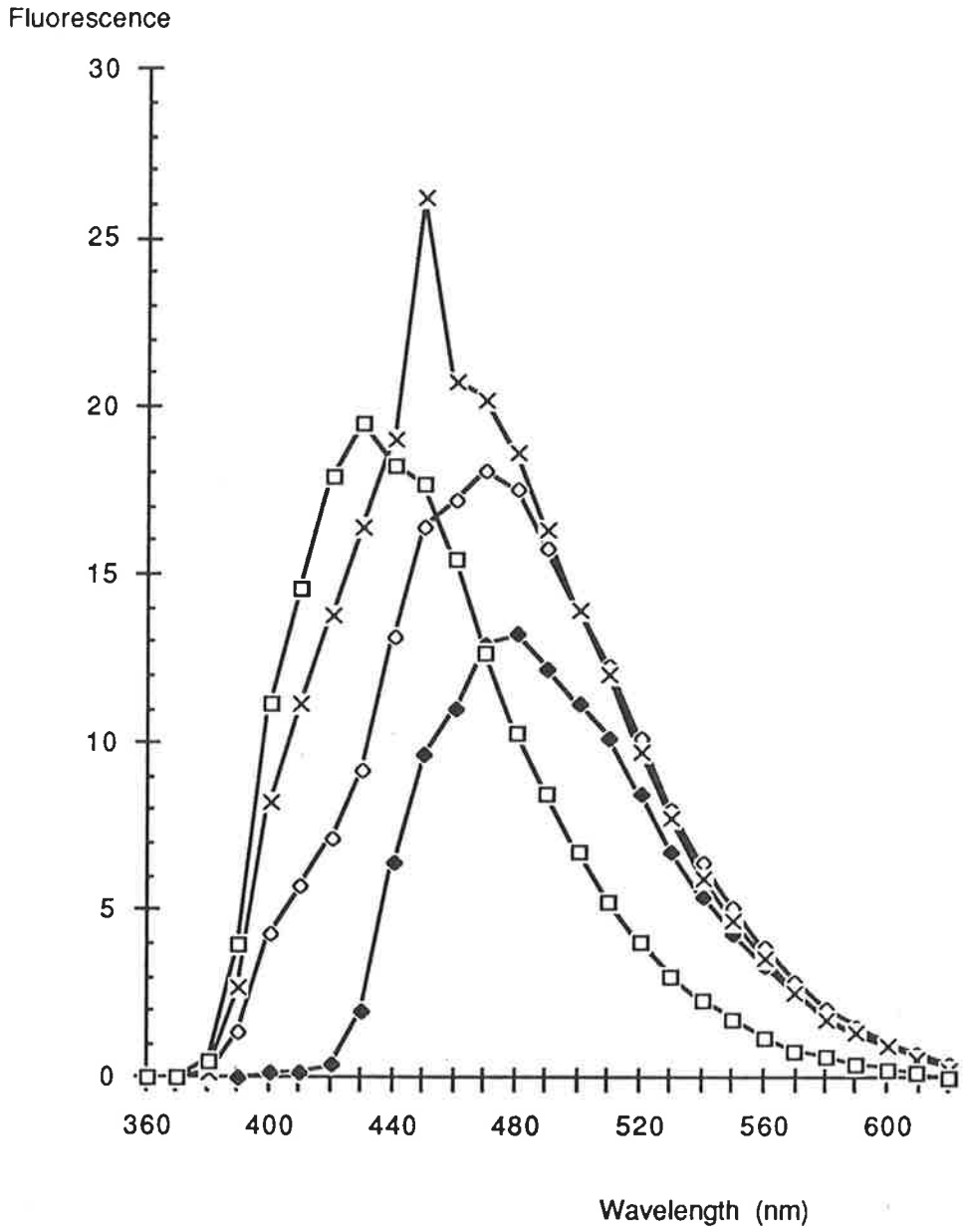


FIGURE 5.3 : Effect of pH on the fluorescence emission spectrum of acridine hydrochloride.

0.05 cm^{-3} of 2×10^{-4} M acridine hydrochloride was added to 2 cm^{-3} of 20 mM citrate buffer, pH 6.17. The emission spectra were recorded with the excitation wavelength set at 347 nm. Small aliquots of concentrated NaOH and HCl were added obtain several different pH values.

(♦-♦-♦) pH 3.17 (o-o-o) pH 5.64 (×-×-×) pH 6.17 (□-□-□) pH 8.04

When acridine hydrochloride solution was added to acidic liposome suspensions in excess neutral buffers the fluorescence emission spectrum had a peak at about 430 nm [Fig. 5.4 i)], indicating that the acridine hydrochloride experienced only the more alkaline environment of the external buffer and very little, if any, was included into the acidic interior. All liposome samples behaved in this way whether they had been prepared by extrusion or had been dialysed, using the Lipoprep device to remove detergent.

Column chromatography of an acidic liposome sample in excess alkaline buffer after acridine hydrochloride was added halved the intensity of the alkaline emission peak at about 430 nm [Fig. 5.4 ii)], and a second chromatography resulted in a spectrum due entirely to the fluorescence of the liposome suspension [Fig. 5.4 iii)].

Alternatively, when the liposome preparation was added to the acridine hydrochloride solution (pH 6.22) [Fig. 5.5], some of the molecules seem to be experiencing an acidic environment. It should be noted that the pH of the bulk solution is lower than in Figure 5.4 and therefore the acidic nature of the fluorescence could reflect this fact. The decrease in intensity suggested that perhaps some molecules were incorporating into the bilayer and not contributing to the emission intensity. This is consistent with reports [132,133] that a related compound, 9-amino-acridine, binds to liposomes, which complicates interpretation of the fluorescence spectra of acridine derivatives.

Fluorescence emission peaks of acridine hydrochloride are quenched by addition of Triton X-100 [Fig. 5.6]. Ethanol and NH_4Cl also cannot be used to equilibrate the pH gradient across the membrane as the former changes the pH of the solution while the latter causes quenching. There is, in fact, considering the above mentioned results, some doubt as to

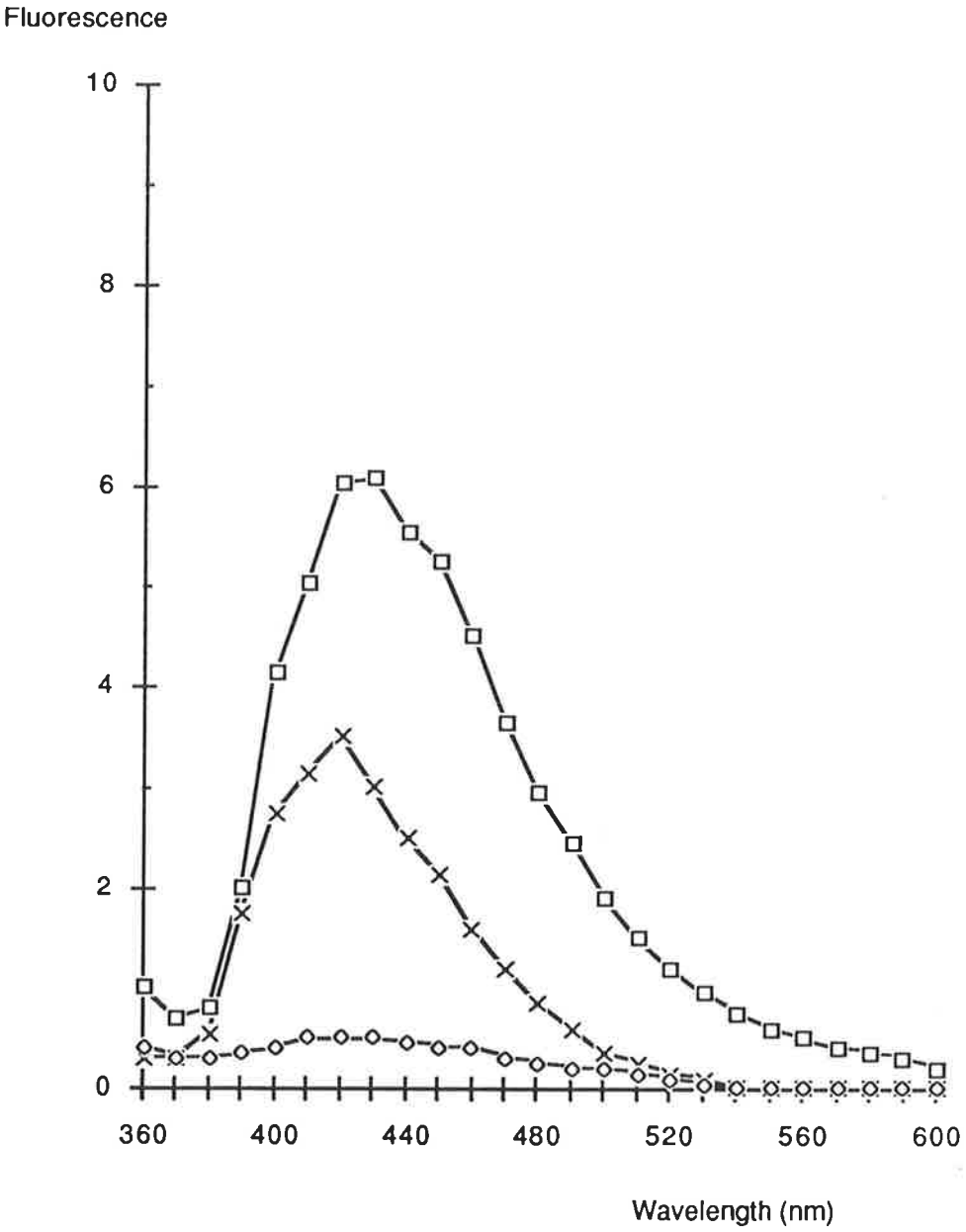


FIGURE 5.4 : Fluorescence emission spectra of acridine hydrochloride with successive passages of solutions of liposomes and acridine hydrochloride solution through a Sephadex G-25 column (using a neutral buffer as eluting solution).
Liposomes prepared from 5 mg cm^{-3} DPPC in glutamate buffer, pH 3.6, and extruded through 100 nm pore size polycarbonate filters.

(i) (□-□-□) liposomes and acridine hydrochloride solution added to a Hepes buffer, pH 7.5

(ii) (×-×-×) after one passage through the Sephadex G-25 column (iii) (o-o-o) after second passage through the Sephadex G-25 column

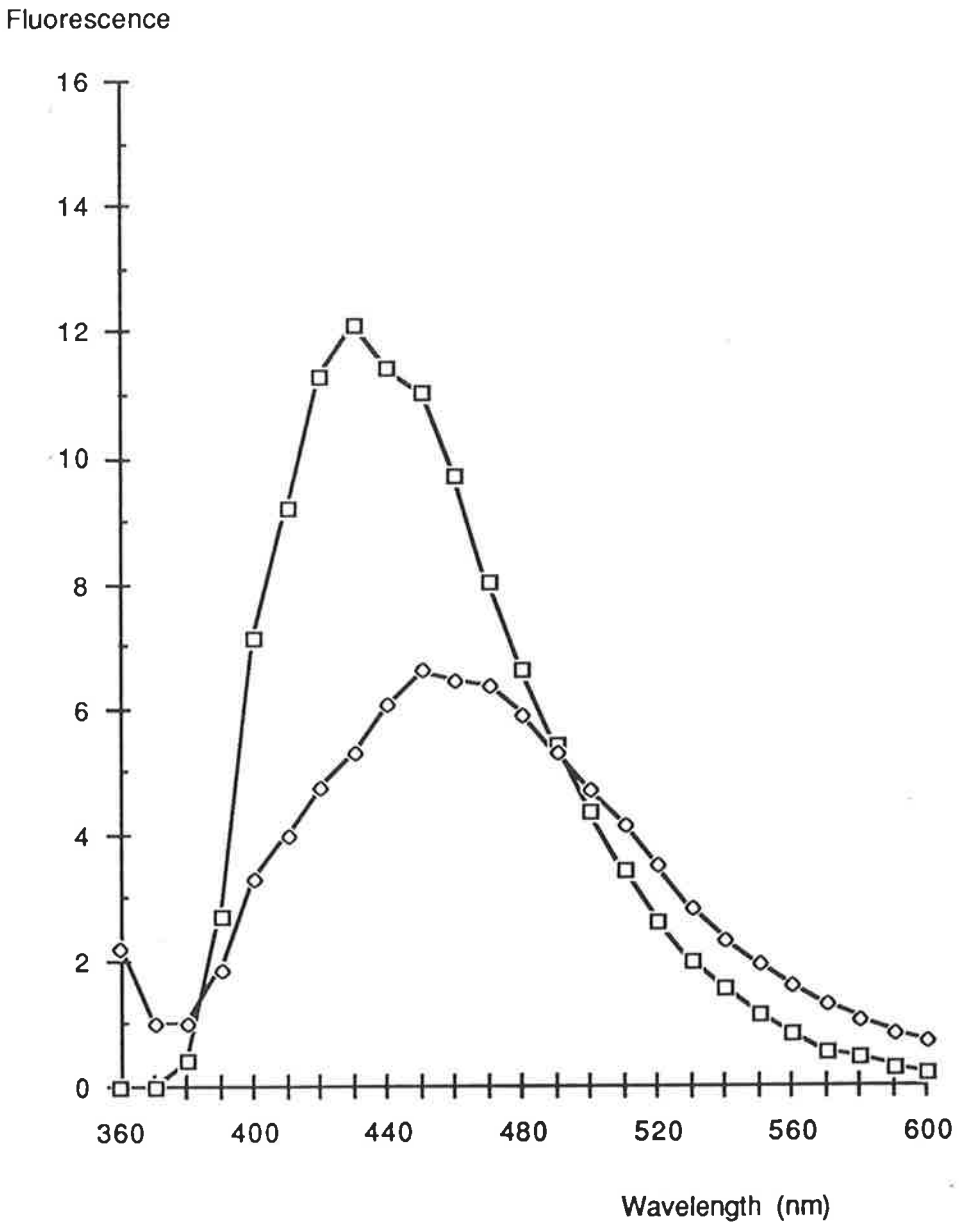


FIGURE 5.5 : Fluorescence emission spectra of acridine hydrochloride with and without the liposome preparation.
Liposomes prepared from 2 mg cm^{-3} asolectin in glutamate buffer, pH 4.82, and extruded through 100 nm pore size polycarbonate filters.

(i) (□-□-□) acridine hydrochloride solution in Hepes buffer, pH 6.22

(ii) (o-o-o) after addition of liposome preparation

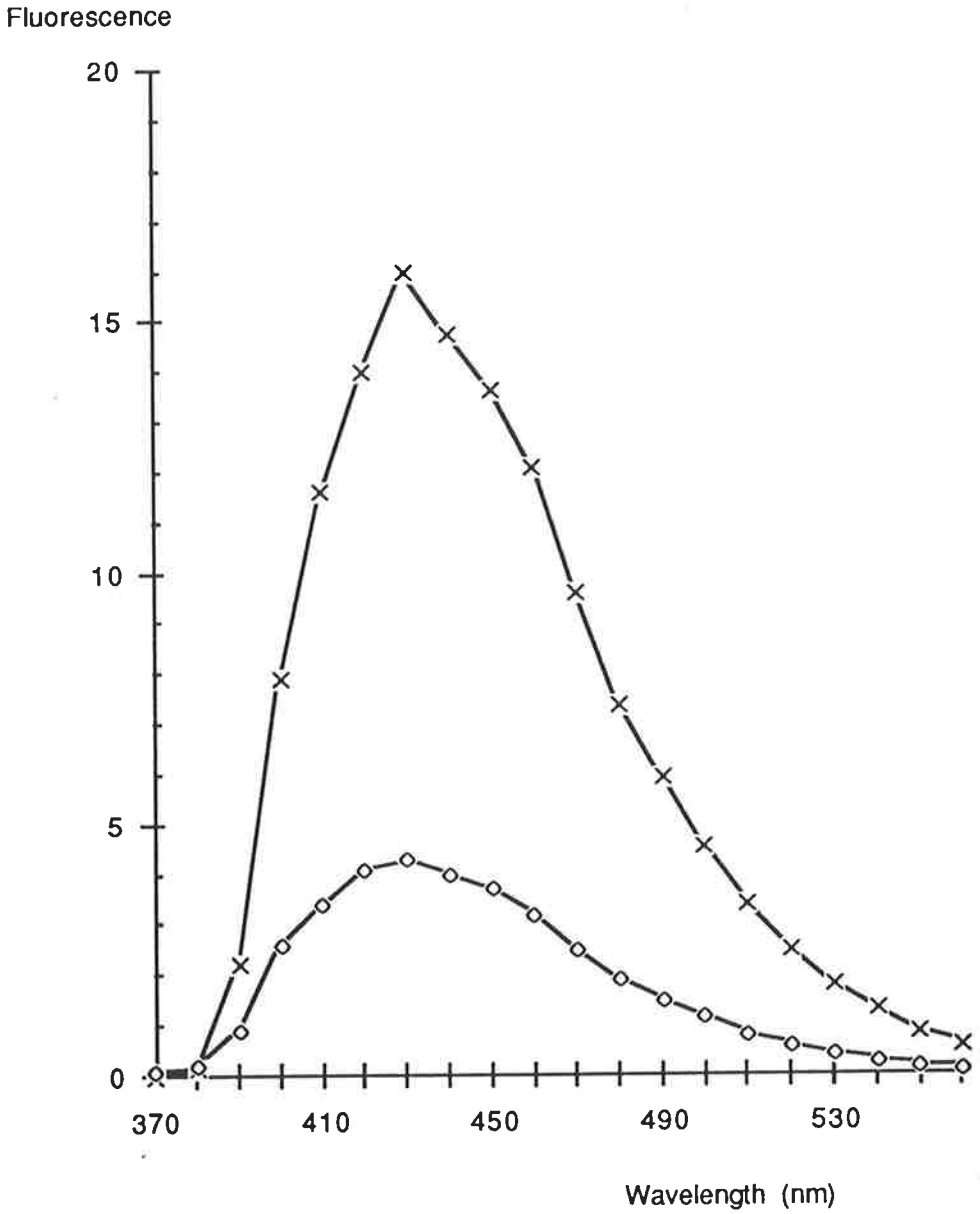


FIGURE 5.6 : Effect of Triton X-100 on the fluorescence emission spectrum of acridine hydrochloride. Emission spectra were recorded with the excitation wavelength set at 350 nm.

(i) (x-x-x) 0.05 cm^3 of 2×10^{-4} M acridine hydrochloride solution in 2 cm^3 of Hepes buffer, pH 7.5

(ii) (o-o-o) after addition of a small amount of concentrated Triton X-100 to cuvette and shaking the solution

whether a pH gradient even exists when the acridine hydrochloride is initially added to the acidic liposomes in an excess of more alkaline buffer.

Due to the above problems with acridine hydrochloride further investigation of the fluorescent amine technique necessitated use of another probe.

5.3.4 Quinine

The effect of pH on the fluorescence emission spectrum of quinine has been determined [107] and [Fig. 5.7] shows the spectra of quinine in both buffers used throughout these experiments. In an acidic environment quinine has an emission peak at about 450 nm (excitation wavelength set at 340/345 nm), while in a more alkaline environment its emission peak is at about 380 nm (excitation wavelength set at 340 nm) and has a lower quantum yield.

When quinine was added to a solution of liposomes prepared in an acidic buffer and then diluted with an excess of more alkaline buffer the emission peak occurred at about 450 nm [Fig. 5.8 i)] indicating that quinine had responded to the pH gradient and was sequestered in the acidic interior of the liposomes.

Self-quenching often occurred, where the properties of the liposomes, the concentration of the probe and the length of time involved all influenced the extent of decrease in the intensity of the peak. It is possible that the internal concentration of the probe may reach such a level that energy transfer between the molecules may cause a decrease in the quantum yield. This phenomenon has been observed previously [134] and an extreme case is illustrated by [Fig. 5.8 ii)]. Once the liposomes were disrupted by addition of Triton X-100, quinine was released into the external buffer and the emission peak was at 390 nm [Fig. 5.8 iii)], indicating that quinine was

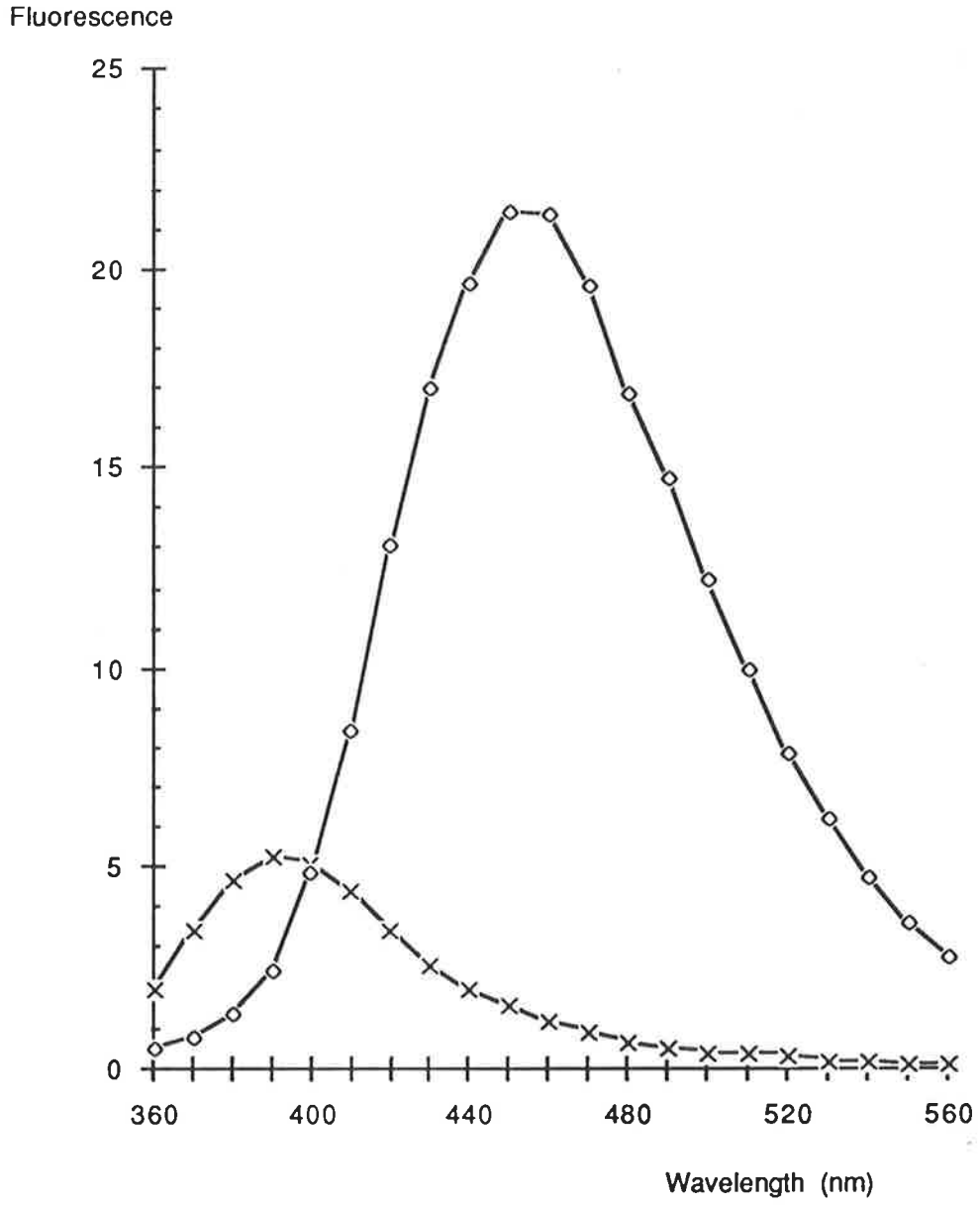


FIGURE 5.7 : Effect of pH on the fluorescence emission spectrum of quinine hydrochloride. Emission spectra were recorded with the excitation wavelength set at 347 nm. The background intensity due to the buffer was subtracted from the spectra.

(i) (×-×-×) 0.1 cm^3 of 25 μM quinine was added to 2 cm^3 of 20 mM citrate buffer, pH 6.0

(ii) (o-o-o) 0.1 cm^3 of 25 μM quinine was added to 2 cm^3 of 20 mM glutamate buffer, pH 3.55

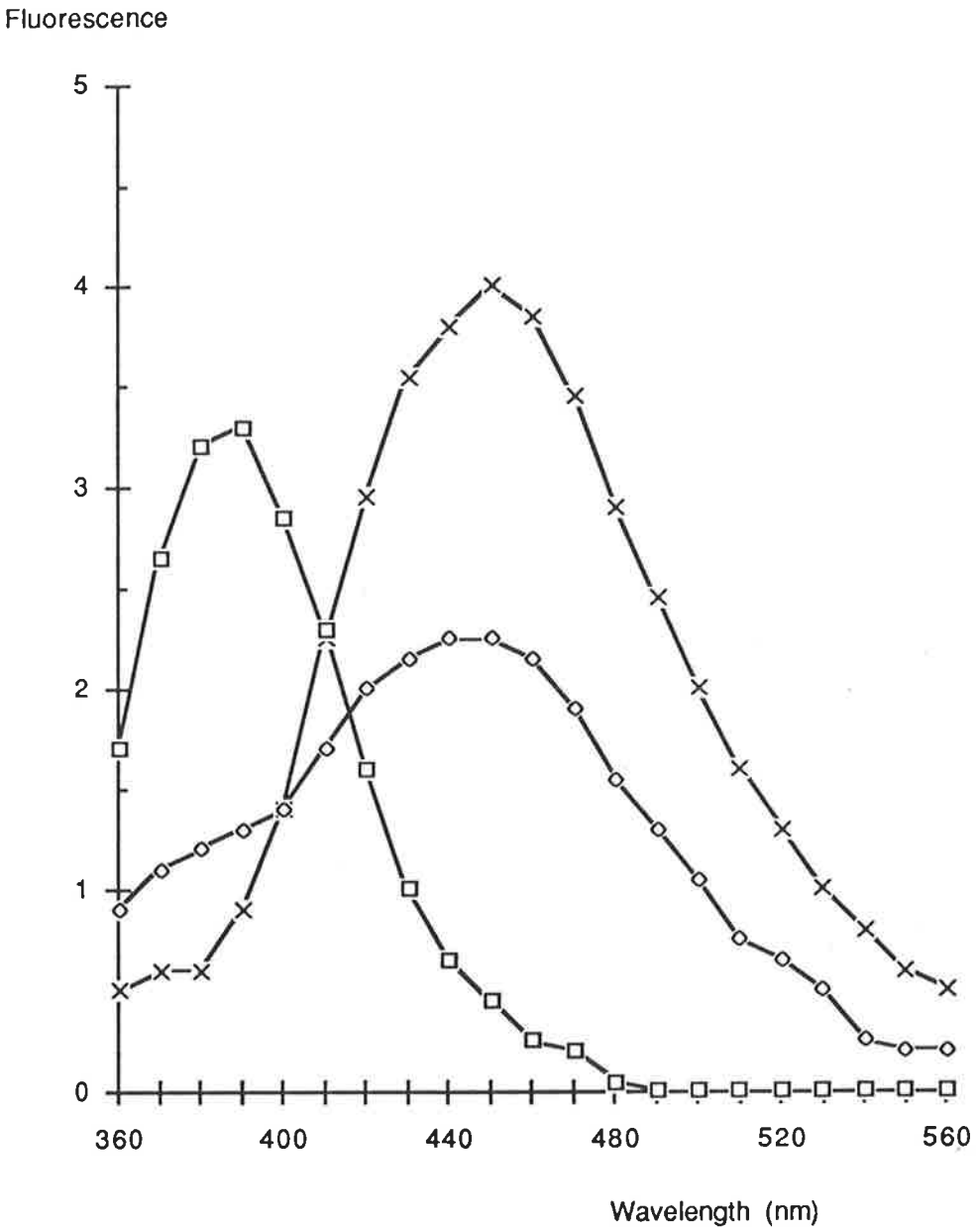


FIGURE 5.8 : Emission spectra were recorded with the excitation wavelength set at 347 nm. The liposomes used were prepared from solutions with asolectin/MEGA-9 molar ratios of 0.15 and 0.24 in 20 mM glutamate buffer, pH 3.6, after at least 17 hours dialysis using the Lipoprep device.
The background intensity due to the buffer was subtracted from the spectra.

(i) (×-×-×) after addition of quinine to acidic liposomes in alkaline buffer

(ii) (o-o-o) self-quenching of acidic peak after solution left standing for 1 hour

(iii) (□-□-□) after addition of Triton X-100 and standing for 2 hours before recording spectrum

experiencing an alkaline environment. Triton X-100 had no effect on the intensity of fluorescence emission of quinine over the wavelengths used and did not alter the pH of the solutions.

Addition of more quinine to a solution of acidic liposomes in a more alkaline buffer already showing quinine to be in an acidic environment resulted in two observations: i) a decrease in the intensity of the peak at about 450 nm and a concomitant increase in the alkaline peak at about 390 nm; or ii) a large increase in the acidic peak incorporating a small increase in the alkaline environment. The former case corresponds to liposomes already containing a large amount of quinine where self-quenching occurs if more quinine is added to the interior and excess quinine remains in the external buffer, while the latter occurs when liposomes are able to accommodate more quinine molecules in their interior.

While some liposomes prepared using the Lipoprep device showed that quinine added to acidic liposomes in an excess of a more alkaline buffer remained in an acidic environment for about 4 hours with little self-quenching, this was not common. Usually much less time (as little as 15 minutes) elapsed before quinine started to experience a more alkaline environment and the spectra showed emission at 390 nm and very little, if any, at 450 nm.

Equilibrating acidic liposomes in an excess of a more alkaline buffer and then adding quinine resulted in a peak at about 385 nm, similar to that obtained when quinine was added to the more alkaline buffer at the same time as the liposomes and left to stand for the same equilibration time [Fig. 5.9]. Thus it appears to be pH equilibration across the membrane that causes this phenomenon.

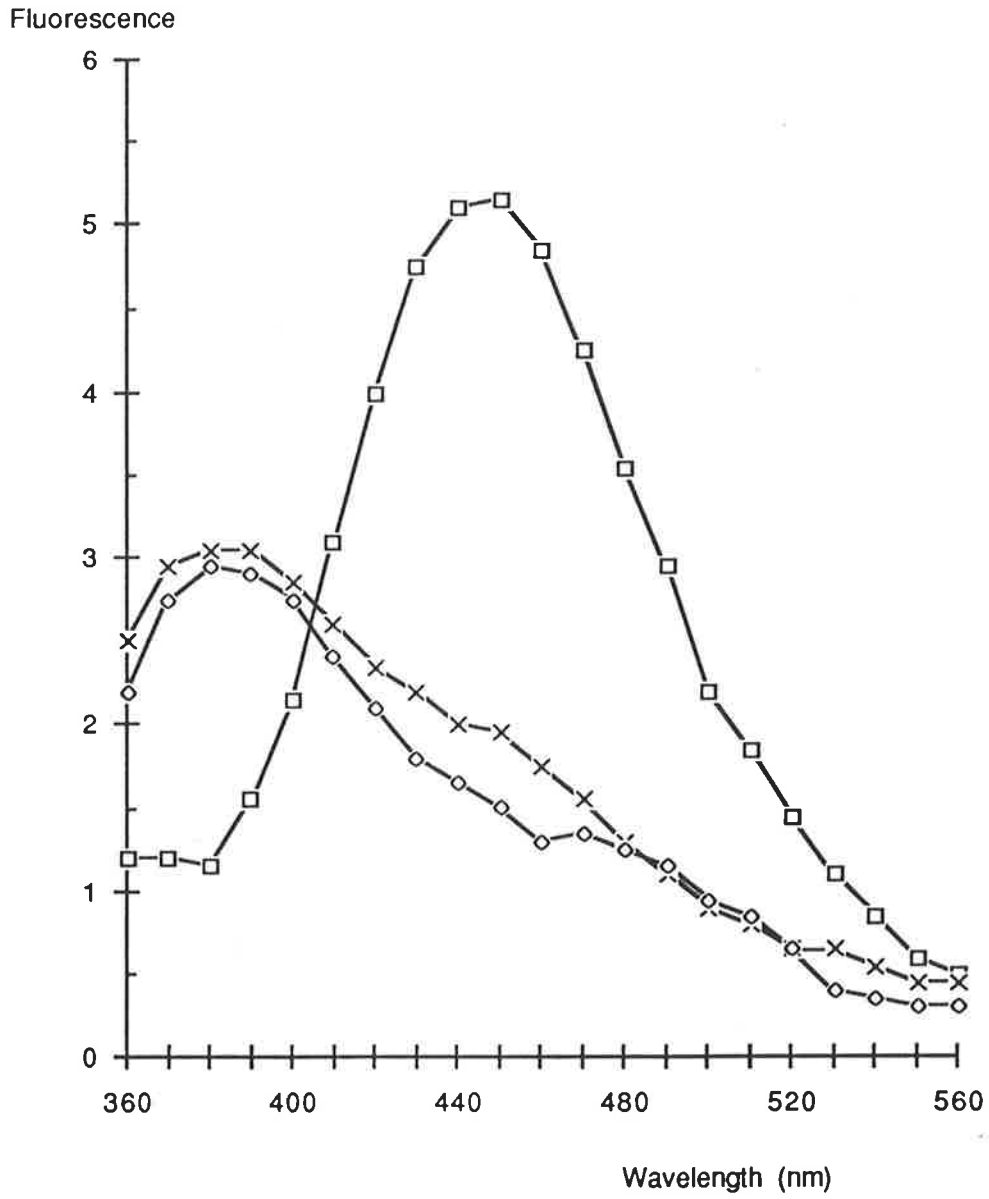


FIGURE 5.9 : Emission spectra were recorded with the excitation wavelength set at 347 nm. The background intensity due to the buffer was subtracted from the spectra.

(i) (□-□-□) after addition of quinine to a solution of acidic liposomes in an excess of a more alkaline buffer

(ii) (×-×-×) the solution after it has been standing for 3 hours

(iii) (o-o-o) acidic liposomes added to an excess of a more basic buffer and left for 3 hours after which time quinine was added (using a different sample of the same preparation) [Liposomes prepared by adding 11.5 mM asolectin and 45.6 mM MEGA-9 (ie. asolectin/MEGA-9 ratio of 0.25) in 20 mM glutamate buffer, pH 3.55, to the Lipoprep device and dialysing for 36 hours.]

Values determined for the permeability of H^+/OH^- through the bilayer membranes of liposomes (where bilayer thickness assumed to be 5 nm range from 10^{-3} cm s^{-1} to 10^{-9} cm s^{-1} (that is, 10^4 nm s^{-1} to 10^{-2} nm s^{-1}) [135,136,137,138]. Differing permeability values probably resulted from minor impurities such as residual solvent or detergent remaining in the membranes after preparation.

Liposomes prepared only one or two days before measurements were performed were more likely to hold a pH gradient (inside acidic) for a reasonable length of time.

While very little detergent is expected to remain in the liposomes prepared by detergent dialysis using the Lipoprep device, it could be sufficient to modify the permeability properties of the phospholipid bilayers [117,118].

Experiments using extruded liposomes were carried out for comparison and showed that quinine only experienced an alkaline environment and was not moving into the acidic interior of the liposomes. The only concession to this behaviour was the observation of a broad peak showing emission in both alkaline and acidic environments which, after a short time, reverted to a larger peak at 380 nm [Fig. 5.10]. Additional aliquots of quinine just increased the intensity of the peak at the lower wavelength.

Liposomes were also prepared in acidic buffers containing quinine and column chromatography used to remove the quinine from the external buffer. These samples behaved similarly to those prepared without quinine, with the spectra usually showing some emission around 450 nm which disappears after a short time leaving only a peak at about 380 nm.

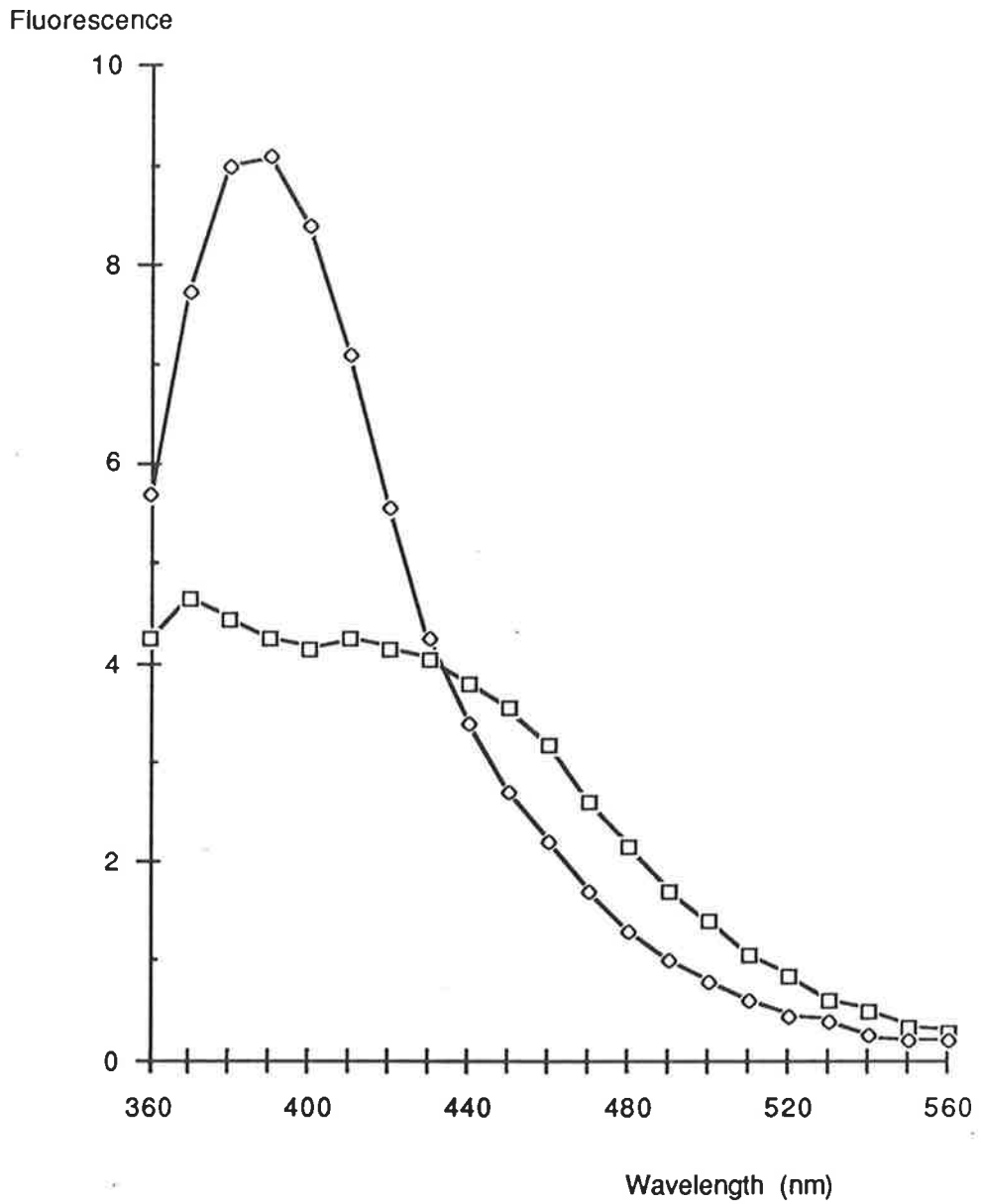


FIGURE 5.10 : Fluorescence emission spectra of quinine with LUVETs.
Liposomes prepared from 2.5 mg cm^{-3} of asolectin in 20 mM glutamate buffer, pH 3.6, using the Rapid Extruder. These acidic liposomes were added to an excess of 20 mM Hepes buffer at pH 7.5.
Spectra were recorded with the excitation wavelength set at 347 nm. The background intensity due to the buffer was subtracted from the spectra.

(i) (□-□-□) after addition of 0.1 cm^3 of 2.5×10^{-5} M quinine solution

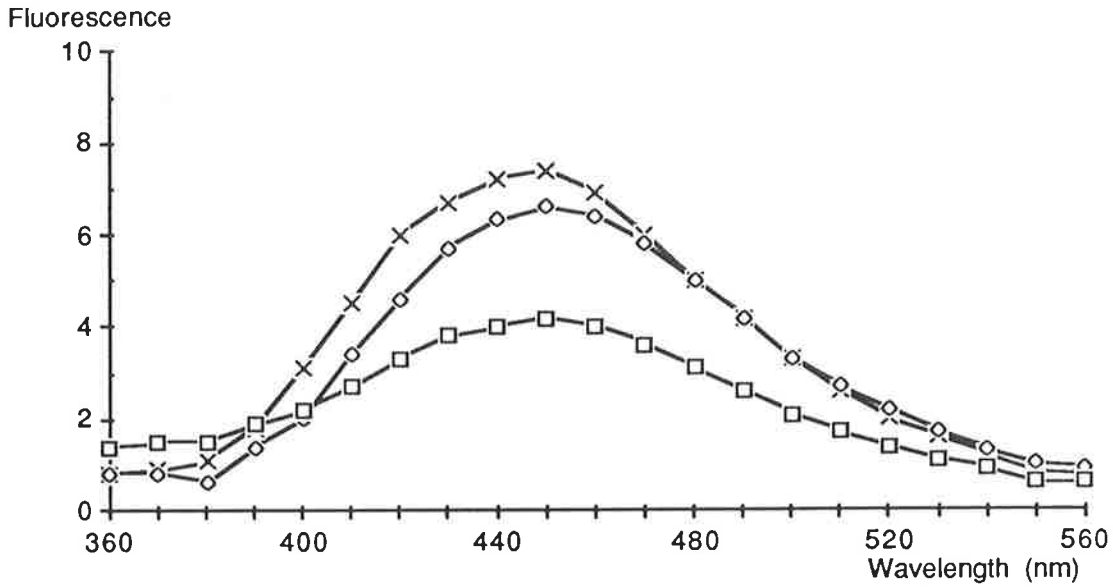
(ii) (o-o-o) after allowing solution to stand for 30 minutes at room temperature

In spite of the above problems with interpreting the resulting spectra it was still considered worthwhile investigating the effects of an applied field. Preliminary tests were carried out using those liposomes, prepared by detergent removal, that exhibited stable pH gradients over a reasonable time. Control and test samples were taken from the same preparation and their spectra analysed together. Most samples exposed to radiofrequency radiation exhibited a larger decrease in the emission around 450 nm and a larger increase in that around 390 nm than that observed for the control samples [Fig. 5.11].

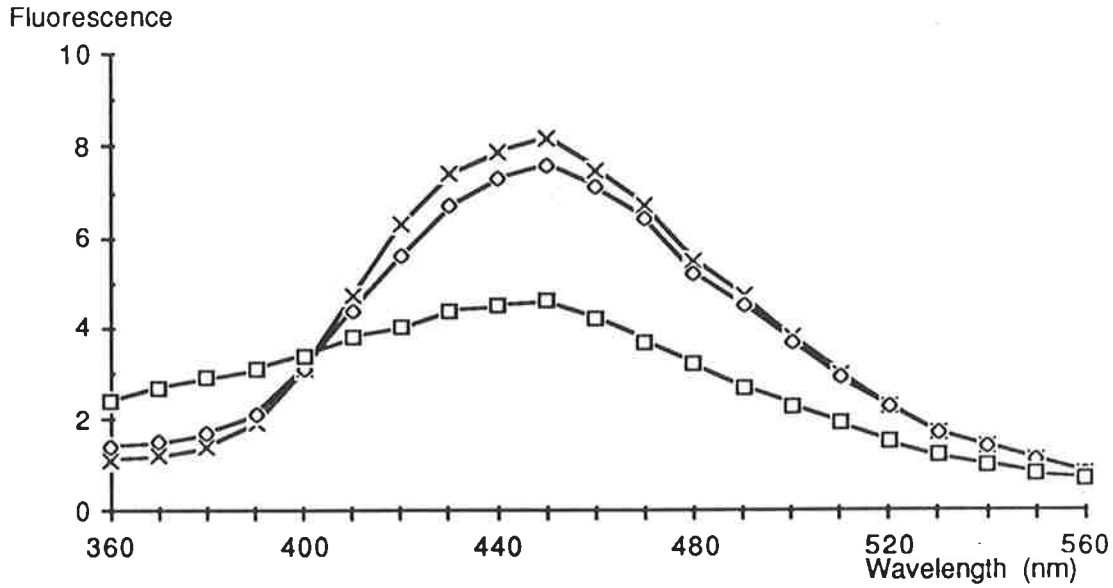
The temperature of the solution in the cuvette was monitored using a thermocouple. During exposure, the temperature of the solution changed from 23° C to about 45° C returning to about 27° C as soon as the field was removed. Mixing the solution by bubbling gas through it or using a Teflon magnetic stirrer would have minimised this temperature excursion. As asolectin was the lipid involved, all temperatures were above the phase transition temperature of -(10°-15°) C and, therefore less likely to affect the permeability of the bilayer structure.

Light scattering measurements were carried out at all stages of the exposure experiments. Application of a radiofrequency field caused no noticeable change in the diameter size of the liposomes but did seem to be consistent with an increase in the polydispersity of the solutions. This suggests that release of the solute occurs via the formation of pores rather than complete disruption.

Addition of Triton X-100 immediately caused an increase in the permeability of liposomes but light scattering measurements showed little change in the diameter size, as extra time was needed to break down and completely disrupt the liposomal structures. An equilibration time of about 30 minutes (depending on the amount added and the system involved)



(a)



(b)

FIGURE 5.11 : Effect of radiofrequency radiation on the fluorescence emission spectrum of quinine which has been included into liposomes.
Liposomes prepared by adding 12.1 mM asolectin amd 51 mM MEGA-9 (ie. lipid/detergent molar ratio of 0.24) in 20 mM glutamate buffer, pH 3.62, to the Lipoprep device and dialysing for 17 hours.
Emission spectra were recorded with the excitation wavelength set at 347 nm. The background intensity due to the buffer and liposomes was subtracted from the spectra.

(a) Control sample : (×-×-×) after addition of quinine to acidic liposomes in excess alkaline buffer (o-o-o) after solution left standing for 1 hour at room temperature

(□-□-□) after solution left standing for 90 more minutes

(b) Test sample : (×-×-×) after addition of quinine to acidic liposomes in excess alkaline buffer (o-o-o) after solution left standing for 1 hour at room temperature

(□-□-□) after solutionexposed to radiofrequency radiation for 90 minutes

changed monodisperse solutions of LUVs into very polydisperse solutions of much smaller dimensions (approximately 20 nm diameter).

Quinine and its isomer, quinidine, have been shown [139] to have a fluidising effect on the bilayer membrane of sonicated PC liposomes, which increases the release of drugs trapped in their aqueous interiors. It was found that 5 mM quinine was sufficient to substantially increase the release of the drugs, even when the liposomes were above T_m, the phase transition temperature, of PC. This need not be a concern in this work, as the concentrations of quinine used are much lower and thus less likely to have a perturbing effect on the membrane.

5.3.5 5,6-Carboxyfluorescein

Insufficient amounts of 5,6-CF were trapped in the aqueous interior of liposomes prepared from lipid/detergent mixed micelles after elution through Sephadex G-50 columns. However, extrusion of solutions containing lipids and 5,6-CF in neutral buffers resulted in very monodisperse populations of LUVs (diameter size about 120 nm). Concentrations of up to 20 mM 5,6-CF were possible in the interior of the liposomes and column chromatography (using Sephadex G-50 columns) removed the dye from the external buffer.

Preliminary experiments showed that this system would allow unambiguous results to be obtained on whether or not the application of fields would perturb the membrane sufficiently to allow the release of 5,6-CF. The advantage of this system is that it has been used extensively to measure the stability of liposomes, therefore its behaviour is well characterised.

5.4 *CONCLUSIONS*

Interest in the effects of electromagnetic fields on human health is increasing and many studies have been undertaken recently which investigate the link between exposure and illnesses, such as depression [140,141,142], fetal death [143,144] and childhood cancer [145]. Other publications address the general hazards [146] and mechanisms [147,148] of the fields. There is a recognised need for more quality epidemiological studies, but the problem is what to study - should it be maximum field strengths, changes in field strengths, average exposure, electric and/or magnetic fields, specific combinations of frequency and intensity of fields or induced currents?

Reba Goodman of Columbia University and Ann Henderson of the City University of New York have undertaken preliminary (but reproducible) tests which indicate that electromagnetic fields indirectly stimulate genes and thereby alter the proteins cells produce [149]. A field of 60 Hertz (no stronger than that produced near many domestic appliances) was found to be a strong promoter of gene transcription, and 45 Hertz had even greater effect. With more experiments of this type, involving biomembranes and also characterised model membranes, more relevant epidemiological studies can be designed and our knowledge of the effects of electromagnetic fields will be increased.

Further studies carried out at Chalmers University using the liposomes prepared and characterised by the method presented here [150] have given conclusive evidence that the permeability of liposomes has indeed been affected after exposure to a field. Extrusion was used as the method of preparation in order to ensure that factors such as residual detergent or organic solvent would not affect the stability and properties of the liposomes.

The lipid used was PC (phosphatidylcholine) and light scattering was used to determine that the liposomes had an average diameter of 185±10 nm. The method of preparing the liposomes, with the fluorescent dye (5,6-CF) trapped in the aqueous interior but absent in the external buffer, was as described earlier in this work.

Control samples were heated to a temperature higher than that reached by the irradiated samples as a result of the absorption of microwave irradiation, with optical fibres used to monitor the temperature of the solutions. Exposure of the liposomes to microwave fields took place above the phase transition of PC, eliminating thermal effects as a possible cause of the increase in the permeability of membranes.

Therefore the model system presented and characterised here is suitable for investigating the effects of electromagnetic fields and continuation of this study using different fields is desirable.

APPENDIX I - Lipids Used

Asolectin was the most commonly used lipid. It was obtained commercially as a soybean product and purified [51]. The composition of asolectin is reported to be 28% phosphatidylcholine, 25.6% phosphatidylethanolamine, 14.4% phosphatidylinositol, 8.8% lysophosphatidylcholine, 6.7% cardiolipin, 3.7% lysophosphatidylethanolamine, 3.6% lysophosphatidylinositol, 3% phosphatidylglycerol, 2.9% phosphatidylserine, and 3.3% unidentified phospholipids [52]. Molecular weight of asolectin is assumed to be 800 $gmol^{-1}$. The phase transition temperature was taken to be similar to egg yolk lecithin, that is, -10° to -15° C.

DL-α-Dipalmitoylphosphatidylcholine (DPPC) was obtained from Sigma Chemical Co. and was 99% pure. The phase transition of DPPC is determined, by fourier transform infrared spectroscopy, to occur at 41.5° C, with a pretransition range from 36° C to 38° C [53].

Both lipids used were stored as solids at -15° C under an atmosphere of nitrogen.

APPENDIX II - Critical Micelle Concentration of Detergents

Critical Micelle Concentrations (CMC) for the various detergents used in the preparation of liposomes.

DETERGENTS	CRITICAL MICELLE CONCENTRATION	REFERENCE
n-octylglucoside	25 mM in H_2O, at 25° C (NaCl lowers the CMC)	151
	(17.4 - 25) mM, in solutions of varying ionic strength	152
sodium cholate	3 mM in 0.15 M NaCl, pH 7-9	152
MEGA 8	60 mM, at 25° C	153
MEGA 9	25 mM	97
MEGA 10	7 mM	97
CHAPS	4 mM, at 25° C	153
Triton X-100	0.2 mM, at 25° C	153

APPENDIX III - Light Scattering Results

The following tables summarise the results of the light-scattering measurements on liposome preparations in the form of two quantities :
i) the mean liposome diameter size (in nm), and
ii) the degree of polydispersity given as the normalised variance of liposome diameter about the mean.

For both quantities the values given represent the results of several separate determinations (typically 4) on a given sample, where the range indicates the total spread of values measured.

When the Q factor (ie. the polydispersity factor) was not much larger than 0.05 (ie. considered homogeneous) this was also consistent with good agreement between values for diameter calculated at all scattering angles ie. 90°, 60° and 45°. Therefore, even at lower angles where larger structures tend to be overemphasised, approximately the same value was determined for the same sample.

APPENDIX III

TABLE I : The results of light scattering measurements are shown. All liposomes were prepared with asolectin as the lipid (at a concentration of 12.5 mM), and the sonication time was 20 minutes. Concentrations of asolectin solutions were calculated assuming that the M.W. of asolectin is 800 $gmol^{-1}$.

NO. OF DAYS AFTER PREPARATION	BUFFER	SCATTERING ANGLE	DIAMETER SIZE (NM)	POLYDISPERSITY FACTOR
2	20 mM glutamate buffer, pH 2.49	90° 60°	88±4 95±5	0.27±0.03 0.40±0.1
12	20 mM glutamate buffer, pH 2.49	90° 60°	86±3 96±5	0.30±0.1 0.35±0.05
6	20 mM glutamate buffer, pH 6	90° 60°	65±4 77±5	0.23±0.03 0.35±0.05

TABLE II : Liposomes prepared using the Lipoprep to remove the detergent. All preparations were made from mixed
micellar solutions of asolectin with 32.5 mM sodium cholate in 5 mM potassium phosphate/150 mM KCl buffer, at pH 7.4.

NO. OF DAYS AFTER PREPARATION	CONC. OF ASOLECTIN (mM)	MOLAR LIPID/DETERGENT RATIO	DIALYSIS TIME (Hours)	DIAMETER SIZE (NM)	POLYDISPER-SITY FACTOR
2	12.5	0.38	4	54±2	0.05±0.02
2	12.5	0.38	6	45±2	0.05±0.03
2	12.5	0.38	8	38±2	0.07±0.02
2	12.5	0.38	10	44±2	0.08±0.01
2	12.5	0.38	25	47±2	0.15±0.03
2	6.25	0.19	10	37±1	0.05±0.02
2	6.25	0.19	21	40±2	0.06±0.02
2	12.5	0.38	10	40±2	0.05±0.02
2	25	0.77	10	43±2	0.05±0.02
2	25	0.77	21	44±2	0.06±0.02
3	12.5	0.38	10	40±2	0.06±0.03
3	43.75	1.35	10	51±2	0.05±0.03
3	43.75	1.35	23	50±2	0.07±0.02
3	62.5	1.92	10	61±2	0.07±0.02
3	62.5	1.92	23	59±2	0.07±0.02

TABLE III : Liposomes prepared using the Lipoprep to remove the detergent. All preparations made from mixed micellar solutions of asolectin and n-OG.

NO. OF DAYS AFTER PREPAR-ATION	CONC. OF ASO-LECTIN (mM)	CONC. OF n-OG	MOLAR LIPID-DETERGENT RATIO	BUFFER SOLUTION	DIALYSIS TIME (Hours)	DIAMETER SIZE (NM)	POLYDISPERSITY FACTOR
2	12.5	50	0.25	5 mM	10	90±3	0.07±0.02
3	12.5	50	0.25	phosphate	27.5	87±2	0.04±0.02
3	25	50	0.5	/150 mM KCl	10	59±2	0.04±0.03
3	43.75	50	0.88	buffer	10	46±3	0.06±0.03
3	43.75	50	0.88	pH 8.2	27.5	46±3	0.06±0.03
1	6.25	50	0.13	5 mM	10	136±4	0.06±0.02
1	6.25	50	0.13	phosphate	25	142±6	0.07±0.02
1	12.5	50	0.25	/150 mM KCl	10	90±3	0.07±0.02
1	25	50	0.5	buffer	10	59±2	0.05±0.02
1	43.75	50	0.88	pH 8.2	10	50±3	0.07±0.02
2	6.25	50	0.13	20 mM	10	55±3	0.06±0.03
2	6.25	50	0.13	glutamate	22	53±2	0.06±0.02
2	12.5	50	0.25	buffer,	10	56±3	0.07±0.02
2	12.5	50	0.25	pH 5.0	22	58±2	0.06±0.02
2	25	50	0.5		10	53±2	0.05±0.03
3	6.25	50	0.13	20 mM	10	42±3	0.07±0.03
3	6.25	50	0.13	glutamate	21	43±3	0.07±0.03
3	12.5	50	0.25	buffer,	10	56±3	0.05±0.03
3	12.5	50	0.25	pH 3.5	21	53±3	0.07±0.03
3	25	50	0.5		10	52±3	0.07±0.03

TABLE IV : Liposomes prepared using the Lipoprep to remove the detergent. All preparations made from mixed micellar solutions of asolectin and n-OG in 20 mM glutamate buffer, pH 2.5.

NO. OF DAYS AFTER PREPAR-ATION	CONC. OF ASOLECTIN (mM)	CONC. OF n-OG	MOLAR LIPID-DETERGENT RATIO	DIALYSIS TIME (Hours)	DIAMETER SIZE (NM)	POLYDISPERSITY FACTOR
2	12.5	50	0.25	10	62±2	0.06±0.04
2	12.5	50	0.25	22	62±2	0.06±0.02
2	25	50	0.5	10	90±3	0.07±0.02
2	25	50	0.5	22	56±3	0.06±0.02
2	43.75	50	0.88	10	82±8	0.45±0.05
10	12.5	50	0.25	10	63±1	0.06±0.03
10	12.5	50	0.25	22	55±3	0.07±0.02
1	6.25	50	0.13	10	55±2	0.09±0.02
1	12.5	50	0.25	10	52±2	0.05±0.03
1	25	50	0.5	10	53±3	0.05±0.02
1	12.5	25	0.5	10	55±3	0.05±0.02
1	6.25	37.5	0.17	10	52±3	0.05±0.02
9	12.5	50	0.25	10	58±2	0.06±0.03
3	6.25	50	0.13	10	53±3	0.07±0.02
3	12.5	50	0.25	10	63±2	0.05±0.02
3	12.5	50	0.25	25	62±2	0.06±0.02
3	25	50	0.5	10	102±6	0.08±0.04
3	25	50	0.5	25	116±3	0.12±0.05
1	11.4	141.8	0.08	10	250±8	0.10±0.04
2	11.4	141.8	0.08	34	240±5	0.12±0.04
1	11.4	120	0.095	10	208±2	0.10±0.04
1	11.4	120	0.095	17	233±3	0.07±0.04
2	11.4	120	0.095	34	206±5	0.08±0.03
7	11.4	120	0.095	34	182±2	0.09±0.02

TABLE V : Liposomes prepared using the Lipoprep to remove the detergent. All preparations made from mixed micellar solutions of DPPC and n-OG.

NO. OF DAYS AFTER PREPAR-ATION	CONC. OF DPPC (mM)	CONC. OF n-OG	MOLAR LIPID-DETERGENT RATIO	BUFFER	DIALYSIS TIME (Hours)	DIAMETER SIZE (NM)	POLYDISPER-SITY FACTOR
2	13.62	68.11	0.20	20 mM	8	163±7	0.15±0.1
2	13.62	68.11	0.20	glutamate	10	185±10	0.16±0.1
2	13.62	68.11	0.20	buffer,	21	115±8	0.14±0.03
2	13.62	90	0.15	pH 2.5	10	165±10	0.14±0.05
2	13.62	90	0.15		21	164±6	0.12±0.05
2	27.2	72.8	0.37	20 mM	10	252±10	0.15±0.1
2	27.2	72.8	0.37	glutamate	25	210±5	0.08±0.02
2	27.2	111.2	0.25	buffer,	10	260±8	0.15±0.1
2	27.2	111.2	0.25	pH 3.5	20	210±4	0.08±0.04
2	27.2	111.2	0.25		25	205±4	0.07±0.03

TABLE VI : Liposomes prepared using the Lipoprep to remove the detergent. All preparations made from mixed micellar solutions of asolectin with MEGA-9, in 20 mM glutamate buffer, pH 3.62.

NO. OF DAYS AFTER PREPAR-ATION	CONC. OF ASO-LECTIN (mM)	CONC. OF MEGA-9	MOLAR LIPID-DETERGENT RATIO	DIALYSIS TIME (Hours)	DIAMETER SIZE (NM)	POLYDISPER-SITY FACTOR
1	11.5	45.6	0.25	10	54±2	0.07±0.03
3	11.5	45.6	0.25	36	57±1	0.06±0.03
1	11.5	69.8	0.16	10	50±10	0.20±0.15
3	11.5	69.8	0.16	36	52±2	0.14±0.04
3	11.5	69.8	0.16	44	55±5	0.12±0.04
4	12.1	80.5	0.15	23	58±8	0.12±0.05
4	12.1	80.5	0.15	44	60±6	0.20±0.05
1	12.1	51	0.24	17	88±6	0.17±0.06
4	12.1	51	0.24	23	98±6	0.15±0.04
4	12.1	51	0.24	44	104±6	0.17±0.05
1	12	44.5	0.27	22	54±4	0.08±0.03
2	12	44.5	0.27	48	58±2	0.09±0.03
1	12	54.7	0.22	22	68±4	0.07±0.03
2	12	54.7	0.22	48	64±3	0.08±0.03
2	12	54.7	0.22	48	68±4	0.08±0.02

TABLE VII : Liposomes prepared by extrusion, using the Rapid Extruder.

NO. OF DAYS AFTER PREPARATION	LIPID	CONC. OF LIPID	BUFFER	NO OF FREEZE/THAW CYCLES	NO. OF EXTRUSIONS	DIAMETER SIZE (NM)	POLYDISPERSITY FACTOR
0	asolectin	2.5	1	7	10	91±2	0.06±0.02
2	"	"	"	"	"	90±3	0.05±0.03
3	"	"	"	"	"	88±2	0.06±0.03
1	asolectin	2.5	2	8	8	115±3	0.06±0.02
0	asolectin	2.5	3	9	BEFORE	225±5	0.15±0.1
"	"	"	"	"	10	126±3	0.05±0.02
0	asolectin	2.5	4	10	BEFORE	268±8	0.14±0.05
"	"	"	"	"	10	124±4	0.06±0.03
13	"	"	"	"	"	122±4	0.07±0.01
1	asolectin	2.5	4+quinine	10	BEFORE	244±8	0.12±0.03
"	"	"	"	"	10	119±2	0.06±0.02
"	"	"	"	"	AFTER ELUTION	117±2	0.06±0.02
0	asolectin	2.5	4+quinine	10	BEFORE	241±4	0.16±0.02
"	"	"	"	"	10	120±2	0.05±0.03

TABLE VII : [CONT.]

NO. OF DAYS AFTER PREPARATION	LIPID	CONC. OF LIPID	BUFFER	NO OF FREEZE/THAW CYCLES	NO. OF EXTRUSIONS	DIAMETER SIZE (NM)	POLYDISPER-SITY FACTOR
0 "	asolectin "	2.5 "	4 "	10 "	BEFORE 10	280±40 121±2	0.13±0.02 0.06±0.02
0	asolectin	2.5	5	10	10	114±3	0.06±0.02
6	asolectin	2.5	6	10	10	141±2	0.06±0.02
1 11	asolectin "	12.5 "	7+$Ru(bipy)_3^{2+}$ "	10 "	10 "	111±2 111±1	0.05±0.02 0.04±0.02
2 "	DPPC "	12.5 " "	7+$Ru(bipy)_3^{2+}$ "	10 "	10 AFTER ELUTION	174±10 140±3	0.15±0.04 0.12±0.02
4	asolectin	12.5	7+5,6-CF "	10	10	123±2	0.07±0.02

TABLE VII : [CONT.]

Buffer 1: 20 mM glutamate buffer, pH 3.63.
Buffer 2: 175 mM glutamate buffer + 75 mM KOH, pH 5.19.
Buffer 3: 85 mM glutamate buffer + 40 mM NaOH, pH 3.6.
Buffer 4: 33 mM glutamate buffer + 3 mM KOH, pH 3.64.
Buffer 5: 175 mM glutamate buffer + 150 mM KOH, pH 4.8.
Buffer 6: 50 mM glutamate buffer + 30 mM KOH, pH 4.4.
Buffer 7: 20 mM Hepes buffer, pH 7.3.

NOTE : Concentration of quinine solution added before preparation was 5 μM.
Concentration of acridine hydrochloride solution added before preparation was 0.5 mM.
Concentration of $Ru(bipy)_3^{2+}$ solution added before preparation was 0.7 mM.
Concentration of 5,6-CF solution added before preparation was 20 mM.

REFERENCES

1. Paul D. Fisher, W. A. G. Voss and Mark J. Poznansky: *Bioelectromagnetics* , 2 , 217-225, (1981).

2. J. R. Thomas, E. D. Finch, D. W. Fulk and L. S. Burch: *Ann. N. Y. Acad. Sci.* , 247 , 425-432, (1975).

3. J. R. Thomas, J. Schrot and R. A. Banvard: *Bioelectromagnetics* , 3 , 227-235, (1982).

4. A. Caddemi, C. C. Tamburello, L. Zanforlin and M. V. Torregrossa: *Bioelectromagnetics* , 7 , 359-367, (1986).

5. A. Caddemi and M. V. Torregrossa: Istituto di Elettrotecnica ed Elettronica , Università di Palermo , R. I., 118, (1983).

6. Paul D. Fisher, Mark J. Poznansky and W. A. G. Voss: *Radiat. Res.*, 92 , 411-422, (1982).

7. R. B. Olcerst, S. Belman, M. Eisenbud, W. W. Mumford and J. R. Rabinowitz: *Radiat. Res.*, 82 , 244-256, (1980).

8. S. F. Cleary, F. Garber and L. M. Liu: *Bioelectromagnetics* , 3 , 453-466, (1982).

9. Robert P. Liburdy and Paul F. Vanek, Jr.: *Radiat. Res.*, 102 , 190-205, (1985).

10. Maimon M. Cohen, Ann Kunska, Jacqueline A. Astemborski, Duncan McCulloch and David A. Paskewitz: *Bioelectromagnetics* , 7 , 415-423, (1986).

11. Nancy Wertheimer and Ed Leeper: *Am. J. Epidemiol.*, 109(3), 273, (1979).

12. Nancy Wertheimer and Ed Leeper: *International J. Epidemiol.*, 11(4), 345-355, (1982).

13. Lennart Tomenius: *Bioelectromagnetics* , 7 , 191-207, (1986).

14. R. P. Blackwell: *Bioelectromagnetics* , 7 , 425-434, (1986).

15. A. Bellossi: *Bioelectromagnetics* , 7 , 381-386, (1986).

16. C. F. Blackman, S. G. Benane, D. E. House and W. T. Joines: *Bioelectromagnetics* , 6 , 1-11, (1985).

17. John R. Thomas, John Schrot and Abraham R. Liboff: *Bioelectromagnetics* , 7 , 349-357, (1986).

18. S. M. Bawin and W. R. Adey: *Proc. Natl. Acad. Sci., U. S. A.*, 73 , 1999-2003, (1976).

19. C. F. Blackman, S. G. Benane, L. S. Kinney, W. T. Joines and D. E. House: *Radiat. Res.*, 95 , 510-520, (1982).

20. C. F. Blackman, S. G. Benane, J. R. Rabinowitz, D. E. House and W. T. Joines: *Bioelectromagnetics* , 6 , 327-337, (1985).

21. J. P. Sheridan, B. P. Gaber, F. Cavatorta and P. E. Schoen: "Molecular level effects of microwaves on natural and model membranes: A Raman Spectroscopic Investigation.", in *Abstract : National Radio Science Meeting and Bioelectromagnetic Symposium* , (L. S. Taylor and A. Y. Cheung, eds.), Seattle, Univ. of Washington, pg. 468, (1979).

22. John Allis and Barbara L. Sinha: *Bioelectromagnetics* , 3 , 323-332, (1982).

23. Robert P. Liburdy and Richard L. Magin: *Radiat. Res.* , 103 , 266-275, (1985).

24. D. Papahadjopoulos: *Ann. N. Y. Acad. Sci.* , 308 , 367-368, (1978).

25. L. Saunders, J. Perrin and D. B. Gammack: *J. Pharm. Pharmacol.*, 14 , 567-572, (1962).

26. M. B. Abramson, R. Katzman and H. P. Gregor: *J. Biol. Chem.*, 239 , 70-76, (1964).

27. A. D. Bangham, M. M. Standish and J. C. Watkins: *J. Mol. Biol.*, 13 , 238-252, (1965).

28. Schmuel Batzri and Edward D. Korn: *Biochim. Biophys. Acta* , 298 , 1015-1019, (1973).

29. M. J. Hope, M. B. Bally, L. D. Mayer, A. S. Janoff and P. R. Cullis: *Chem. Phys. Lipids* , 40(2-4), 89, (1986).

30. G. Gregoriadis and A. C. Allison (Eds.): "*Liposomes in Biological Systems* ", John Wiley and Sons, Ltd., (1980).

31. D. A. Tyrrell, T. D. Heath, C. M. Colley and Brenda E. Ryman: *Biochim. Biophys. Acta* , 457 , 259-302, (1976).

32. K. Kreuschner: in "*Liposomes as Drug Carriers* ", Symposium Tübingen, October, 1984, (K. H. Schmidt, ed.), Georg Thieme Verlag, Stuttgart, (1986).

33. Francis Szoka, Jr. and Demetrios Papahadjopoulos: *Ann. Rev. Biophys. Bioenerg.*, 9 , 467-508, (1980).

34. Y. Barenholz; D. Gibbes, B. J. Litman, J. Goll, T. E. Thompson and F. D. Carlson: *Biochemistry* , 16(12), 2806-2810, (1977).

35. John Bramhall: *Biochemistry* , 25 , 3479-3486, (1986).

36. G. Cevc, J. M. Seddon and R. Hartung: in "*Liposomes as Drug Carriers* ", Symposium Tübingen, October, 1984, (K. H. Schmidt, ed.), Georg Thieme Verlag, Stuttgart, (1986).

37. M. L. Jackson, C. F. Schmidt, D. Lichtenberg, B. J. Litman and A. O. Albert: *Biochemistry* , 21 , 4576-4582, (1982).

38. Ching-hsien Huang: *Biochemistry* , 8(1), 344-352, (1969).

39. D. Papahadjopoulos and W. J. Vail: *Ann. N. Y. Acad. Sci.* , 308 , 259-266, (1978).

40. D. W. Deamer: *Ann. N. Y. Acad. Sci.* , 308 , 250-258, (1978).

41. Francis Szoka, Fred Olson, Timothy Heath, William Vail, Eric Mayhew and Demetrios Papahadjopoulos: *Biochim. Biophys. Acta* , 601 , 559-571, (1980).

42. Y. Barenholz, S. Amselem and D. Lichtenberg: *FEBS Letters* , 99 , 210-214, (1979).

43. M. J. Hope, M. B. Bally, G. Webb and P. R. Cullis: *Biochim. Biophys. Acta* , 812 , 55-65, (1985).

44. S. Almog, T. Kushir, S. Nir and D. Lichtenberg: *Biochemistry* , 25 , 2597-2605, (1986).

45. J. Brunner, P. Skrabal and H. Hauser: *Biochim. Biophys. Acta* , 455 , 322-331, (1976).

46. H. G. Enoch and P. Strittmatter: *Proc. Natl. Acad. Sci., U. S. A.*, 76 , 145-149, (1979).

47. W. G. Gerritsen, A. J. Verkleij, R. F. Zwaal and L. L. M. van Deenen: *Eur. J. Biochem.*, 85 , 255-261, (1978).

48. J. Philippot, S. Mutaftschiev and J. P. Liautard: *Biochim. Biophys. Acta* , 734 , 137-143, (1983).

49. Y. Kagawa and E. Racker: *J. Biol. Chem.*, 246 , 5477-5487, (1971).

50. Manfred H. W. Milsmann, Reto A. Schwendener and Hans Georg Weder: *Biochim. Biophys. Acta* , 512 , 147-155, (1978).

51. Same asolectin as used in Per-Eric Thörnström; Bassam Sou; Lars Arvidsson; and Bo Malmström, *Chemica Scripta* , 24 , 230-235, (1984).

52. R. Letters: *Biochem. J.*, 93 , 313-316, (1964).

53. David G. Cameron, Hector L. Casal and Henry H. Mantsch: *Biochemistry* , 19 , 3665-3672, (1980).

54. C. Huang and T. E. Thompson: *Methods in Enzymology* , 32 , 485-489, (1975).

55. R. A. Klein: *Biochim. Biophys. Acta* , 210 , 486, (1970).

56. H. O. Hauser: *Biochem. Biophys. Res. Commun.*, 45(4), 1049-1055, (1971).

57. D. M. Small: *Gastroenterology* , 52 , 607, (1967).

58. Norman Mazer, George B. Benedek and Martin C. Carey: *Biochemistry* , 19(4), 601-615, (1980).

59. D. M. Small, M. C. Bourges and D. G. Dervichian: *Biochim. Biophys. Acta* , 125 , 563-580, (1966).

60. M. C. Carey and D. M. Small: *Ann. J. Med.*, 49 , 590-608, (1970).

61. N. A. Mazer, R. F. Kwasnick, M. C. Carey and G. B. Benedek: in "*Micellisation, Solubilisation and Microemulsions* ", (K. L. Mittal, ed.), Vol. I, pgs. 383-402, Plenum Press, New York, (1979).

62. Theresa M. Allen, Alice Y. Romans, Henri Kercret and Jere P. Segrest: *Biochim. Biophys. Acta* , 601 , 328-342, (1980).

63. R. A. Schwendener and H. G. Weder: *Biochem. Pharmacol.*, 27 , 2721-2727, (1978).

64. O. Zumbuehl and H. G. Weder: *Biochim. Biophys. Acta* , 640 , 252-262, (1981).

65. R. A. Schwendener, M. Asanger and H. G. Weder: *Biochem. Biophys. Res. Commun.*, 100(3), 1055-1062, (1981).

66. D. W. Deamer and P. S. Uster: in "*Liposomes* ", (Marc Ostro, ed.), pgs. 27-51, Marcel Dekker Inc., New York, (1983).

67. H. G. Weder and O. Zumbuehl: in "*Liposome Technology, Vol. I* ", pgs. 79-107, (G. Gregoriadis, ed.), CRC Press Inc., Boca Raton, FL, (1984).

68. Jacqueline A. Reynolds, Yasuhiko Nozaki and Charles Tanford: *Anal. Biochem.* , 130 , 471-474, (1983).

69. T. M. Allen: in "*Liposome Technology, Vol. I* ", pgs. 109-122, (G. Gregoriadis, ed.), CRC Press Inc., Boca Raton, FL, (1984).

70. Larry T. Mimms, Guido Zampighi, Yasuhiko Nozaki, Charles Tanford and Jacqueline A. Reynolds: *Biochemistry* , 20 , 833-840, (1980).

71. F. Olson, C. A. Hunt, F. C. Szoka, W. J. Vail and D. Papahadjopoulos: *Biochim. Biophys. Acta* , 557 , 9-23, (1979).

72. J. A. Crommelin and E. M. G. Van Bommel: in "*Liposomes as Drug Carriers* ", Symposium Tübingen, October, 1984, (K. H. Schmidt, ed.), Georg Thieme Verlag, Stuttgart, (1986).

73. L. D. Mayer, M. J. Hope, P. R. Cullis and A. S. Janoff: *Biochim. Biophys. Acta* , 817 , 193-196, (1985).

74. George Strauss: in "*Liposome Technology, Vol. I* ", pgs. 197-219, (G. Gregoriadis, ed.), CRC Press Inc., Boca Raton, FL, (1984).

75. J. C. Selser, Y. Yeh and R. J. Baskin: *Biophys. J.*, 16 , 337-356, (1976).

76. J. E. Frederick, T. F. Reed and O. Kramer: *Macromolecules* , 4(2), 242, (1971).

77. D. E. Koppel: *J. Chem. Phys.*, 57 , 4814, (1972).

78. P. N. Pusey, D. W. Schaefer, D. E. Koppel and R. D. Camerini-Otero: *J. Phys. (Paris)* , 33 , C1-163, (1972).

79. F. C. Chen, A. Chrzeszczyk and B. Chu: *J. Chem. Phys.* , 64(8), 3403-3409, (1976).

80. Nicole Ostrowsky and Didier Sornette: in "*Biomedical Applications of Laser Light Scattering* ", (D. B. Sattelle; W. I. Lee; and B. R. Ware, eds.), Elsevier Biomedical Press, (1982).

81. P. Schurtenberger and H. Hauser: *Biochim. Biophys. Acta* , 778 , 470-480, (1984).

82. P. Schurtenberger, N. Mazer and W. Känzig: *J. Phys. Chem.* , 89 , 1042-1049, (1985).

83. Yasuhiko Nozaki, Danilo D. Lasic, Charles Tanford and Jacqueline A. Reynolds: *Science* , 217 , 366-367, (1982).

84. G. Perevucnik, P. Schurtenberger, D. D. Lasic and H. Hauser: *Biochim. Biophys. Acta* , 821 , 169-173, (1985).

85. B. De Kruijff, A. Rietveld and P. R. Cullis: *Biochim. Biophys. Acta* , 600 , 343-357, (1980).

86. L. D. Mayer, M. J. Hope and P. R. Cullis: *Biochim. Biophys. Acta* , 858 , 161-168, (1986).

87. L. D. Bergelson: *Methods Membrane Biol.*, 9 , 275-335, (1979).

88. E. G. Finer, A. G. Flook and H. Hauser: *Biochim. Biophys. Acta* , 260 , 49-58, (1972).

89. Joseph A. N. Zasadzinski: *Biophys. J.*, 49 , 1119-1130, (1986).

90. P. N. Pusey, D. E. Koppel, D. W. Schaeffer, R. D. Camerini-Otero and S. H. Koenig: *Biochemistry* , 13 , 952, (1974).

91. W. Shankland: *Chem Phys. Lipids* , 4 , 109, (1970).

92. R. F. King and R. M. Marchbanks: *Biochim. Biophys. Acta* , 691 , 183-187, (1982).

93. Ari Helenius and Kai Simons: *Biochim. Biophys. Acta* , 415 , 29-79, (1979).

94. Danilo D. Lasic: *Biochim. Biophys. Acta* , 692 , 501-502, (1982).

95. C. Baron and T. E. Thompson: *Biochim. Biophys. Acta* , 382 , 276-285, (1975).

96. James E. K. Hildreth: *Biochem. J.*, 207 , 363-366, (1982).

97. Mitsuya Hanatani, Keiko Nishifuji, Masamitsu Futai and Tomofusa Tsuchiya: *J. Biochem.*, 95 , 1349-1353, (1984).

98. U. Zimmerman: *Biochim. Biophys. Acta* , 694 , 227-277, (1982).

99. Justin Teissie and Tian Yow Tsong: *Biochemistry* , 20 , 1548-1554, (1981).

100. E. Mostafa El-Mashak and Tian Yow Tsong: *Biochemistry* , 24 , 2884-2888, (1985).

101. Lellis F. Braganza, Barry H. Blott, Tessa J. Coe and David Melville: *Biochim. Biophys. Acta* , 731 , 137-144, (1983).

102. M. B. Bally, M. J. Hope, C. J. A. Van Echteld and P. R. Cullis: *Biochim. Biophys. Acta* , 812 , 66-76, (1985).

103. Lawrence D. Mayer, Marcel B. Bally, Michael J. Hope and Pieter R. Cullis: *J. Biol. Chem.*, 260(2), 802-808, (1985).

104. Lawrence D. Mayer, Marcel B. Bally, Michael J. Hope and Pieter R. Cullis: *Biochim. Biophys. Acta* , 816 , 294-302, (1985).

105. L. D. Mayer, M. B. Bally and P. R. Cullis: *Biochim. Biophys. Acta* , 857 , 123-126, (1986).

106. S. Schuldiner, H. Rottenberg and M. Avron: *Eur. J. Biochem.*, 25 , 64-70, (1972).

107. Hon Cheung Lee and John G. Forte: *Biochim. Biophys. Acta* , 601 , 152-166, (1980).

108. J. N. Weinstein, S. Yoshikami, P. Henkart, R. Blumenthal and W. A. Hagins: *Science* , 195 , 489-492, (1977).

109. J. N. Weinstein, R. Blumenthal, S. O. Sharrow and P. A. Henkart: *Biochim. Biophys. Acta* , 509 , 272, (1978).

110. L. D. Leserman, J. N. Weinstein, R. Blumenthal and W. D. Terry, *Proc. Natl. Acad. Sci. U.S.A.*, 77 , 4089, (1980).

111. John N. Weinstein, Evelyn Ralston, Lee D. Leserman, Richard D. Klausner, Paul Dragsten, Pierre Henkart and Robert Blumenthal: in "*Liposome Technology, Vol. III* ", (G. Gregoriadis, ed.), pgs. 183-204, CRC Press, Boca Raton, FL, (1984).

112. Judith Senior and Gregory Gregoriadis: in "*Liposome Technology, Vol. III* ", (G. Gregoriadis, ed.), pgs. 263-282, CRC Press, Boca Raton, FL, (1984).

113. G. Gregoriadis and J. Senior: *FEBS Letters* , 119 , 43, (1980).

114. Peter I. Lelkes: in "*Liposome Technology, Vol. III* ", (G. Gregoriadis, ed.), pgs. 225-246, CRC Press, Boca Raton, FL, (1984).

115. Graciela Ruderman and J. Raul Grigera: *Biochim. Biophys. Acta* , 863 , 277-281, (1986).

116. Masuhara Ueno: *Biochemistry* , 28 , 5631-5634, (1989).

117. K. Inoue and T. Kitagawa: *Biochim. Biophys. Acta* , 426 , 1-16, (1976).

118. Roberta A. Parente and Barry R. Lentz: *Biochemistry* , 23 , 2353-2362, (1984).

119. M. Sila, S. Au and N. Weiner: *Biochim. Biophys. Acta* , 859 , 165-170, (1986).

120. Alicia Alonso, Maria-Angeles Urbaneja, Félix M. Goñi, Francisco G. Carmona, Francisco G. Cánovas and Juan C. Gomez-Fernández: *Biochim. Biophys. Acta* , 902 , 237-246, (1987).

121. Javier-Ruiz, Félix M. Goñi and Alicia Alonso: *Biochim. Biophys. Acta* , 937 , 127-134, (1988).

122. Brenda E. Ryman and D. A. Tyrrell: in "*Essays in Biochemistry* ", Vol. 16, PGS. , published for the Biochemical Society by Academic Press, (1980).

123. Private communication with Tom Redelmeier, Faculty of Medicine, Dept. of Biochemistry, University of British Columbia, Vancouver, B. C., Canada.

124. M. C. Blok, E. C. M. Van Der Neut-Kok, L. L. M. Van Deenen and J. De Gier: *Biochim. Biophys.Acta* , 406 , 187, (1975).

125. A. G. Lee: *Biochim. Biophys. Acta* , 472 , 237, (1977).

126. M. C. Blok, L. L. M. Van Deenen and J. De Gier: *Biochimica et Biophysica Acta* , 433 , 1, (1976).

127. K. Inoue: *Biochim. Biophys. Acta* , 339 , 390, (1974).

128. D. Papahadjopoulos, K. Jacobson, S. Nir and T. Isac: *Biochim. Biophys. Acta* , 311 , 330, (1973).

129. Koji Kano and Janos H. Fendler: *Biochim. Biophys. Acta* , 509 , 289-299, (1978).

130. A. D. Bangham: in "*Progress in Biophysics and Molecular Biology* ", (J. A. V. Butler and D. Noble, eds.), pg. 29, Pergamon Press, Oxford and New York, (1968).

131. J. D. McGivan: in "*The Movement of Ions across Artificial Phospholipid Membranes* ", Ph. D. Thesis, University of Bristol, 1968.

132. J. W. T. Fiolet, E. P. Bakker and K. Van Dam: *Biochim. Biophys. Acta* , 368 , 432-445, (1974).

133. Stephan Grzesiek and Norbert A. Dencher: *Biochim. Biophys. Acta* , 938 , 411-424, (1988).

134. D. W. Deamer, R. C. Prince and A. R. Crofts: *Biochim. Biophys. Acta* , 274 , 323-335, (1972).

135. D. W. Deamer and J. W. Nichols: *Proc. Natl. Acad. Sci. U. S. A.*, 80 , 165, (1983).

136. D. S. Cafiso and W. L. Hubbell: *Biophys. J.* , 44 , 49, (1983).

137. J. Gutknecht: *J. Membrane Biol.*, 82 , 105, (1984).

138. Israel N. Miller: *Bioelectrochem. Bioenergetics* , 19 , 359-369, (1988).

139. Hiroko Nakae and Shozo Asada: *Chem. Pharm. Bull.*, 34(5), 2169-2172, (1986).

140. F. S. Perry and L. Pearl: *Public Health* , 102 , 11-18, (1988).

141. F. S. Perry, L. Pearl and R. Binns: *Public Health* , 103 ,177-180, (1989).

142. D. Dowson, G. T. Lewith, M. Campbell, M. A. Mullee and L. A. Brewster: *Practitioner* , 435-436, 22 April, (1988).

143. N. Wertheimer and E. Leeper: *Bioelectromagnetics* , 7 , 13-22, (1988).

144. N. Wertheimer and E. Leeper: *Am. J. Epidemiol.*, 129 , 220-224, (1989).

145. D. A. Savitz, H. Wachtel, F. A. Barnes, E. M. John and J. G. Tvrolik: *Am. J. Epidemiol.*, 128 , 21-38, (1988).

146. C. W. Smith and S. T. Best: "*Electromagnetic Man : Health and Hazard in the Electrical Environment* ", London, Dent, (1989).

147. A. A. Marino (ed.): "*Modern Bioelectricity* ", New York, Marcel Dekker, (1988).

148. H. Fröhlich: "*Biological Coherence and Response to External Stimuli* ", Heidelberg, Springer, (1988).

149. Tim Beardsley: *Scientific American* , pg. 13, July, (1990).

150. Elisabeth Saalman, Bengt Nordén, L. Arvidsson, Yngve Hamnerius, Per Höjevik, Karen Connell and Tomas Kurucsev: Submitted to *Biochim. Biophys. Acta* in June, (1990).

151. K Shinoda, T. Yamaguchi and R. Hori: *Bull. Chem. Soc. Jpn.*, 34 , 237, (1961).

152. Weder, *et al.*, in "Liposomes as Drug Carriers", Symposium Tübingen. October, (1984).

153. Boehringer Mannheim Biochemica manual.

PART II - PLANAR BILAYER LIPID MEMBRANES

CHAPTER 6 - INTRODUCTION

Historically, it was Overton who was the first to associate lipids with the cellular membrane when he found that lipids or lipid-like substances readily diffused across the membranes [1]. The plasma membrane model proposed by Davson-Danielli [2,3] was based on the results of independent experiments done by Fricke [4] and Gorter and Grendel in the 1920s [5]. This bimolecular lipid leaflet model was later modified by Robertson [6], and is still subject to discussion.

It was recognised that studies of basic, well-defined "synthetic bilayer systems" could provide relatively simple models for the physical chemical characterisations of natural membranes. Early attempts to generate such a lipid bilayer membrane system were made [7,8,9], but it was not until the early 1960s that Mueller, *et al.* [10] first reported the formation of membranous structures, from lipids and proteolipids of bovine brain, and suggested the probable structure of a bimolecular lipid membrane in aqueous solution. It has been assumed that bilayer lipid membranes adopt the 'neat' smectic mesomorphic form. The polar headgroups face outward in contact with the aqueous solution and the non-polar hydrocarbon chains are assumed to be in a liquid state. Lipids and proteolipids extracted from bovine brain were used in further experiments on reconstituted membranes *in vitro* [11,12,13].

In the following discussions, the nomenclature suggested by Montal [14] will be used, where *black lipid films* will be used to describe the Mueller-Rudin-Tien-Wescott bilayers, which are thought to contain solvent. Those bilayers which are "solvent-free", prepared by methods similar to that of Montal and Mueller, are called *lipid bilayers.* Although the term *BLM* has been used extensively, especially by Tien [15], to mean

"black lipid membrane", in this work it instead has the more general meaning of "bilayer (or bimolecular) lipid membrane".

(a) *Black Lipid Membranes*

Adaption of the methods used to prepare soap films permitted the formation of bimolecular lipid membranes in saline solutions [16]. Water-insoluble lipids in chloroform/methanol solutions were spread underwater across frames or loops composed of non-polar materials which are more strongly wetted by the organic solution than by water. The optical appearance of the resulting black films was the complete analogue of soap films. Artificial membranes formed in this way consist of planar lipid bilayers surrounded by a thick annulus of the parent lipid solution called the Plateau-Gibbs border [17]. Since the annulus, or torus, has an average thickness much greater than that of the bilayer, it is assumed that the electrical and permeability properties of the system are determined by the bilayer portion of the film [18]. There have been many reviews published that discuss the formation and properties of these black lipid films [15,19,20,21,22,23]. Black lipid films usually retain some of the solvent used for their formation and have a thickness greater than the thickness of the bilayer portion of a biological membrane [24]. It was therefore recognised that "the black lipid film is not precisely a lipid bilayer since from the nature of the system in which it is formed, it must, in general, contain some lipid solvent" [23].

White [25,26] and others [27,28,29,30] have shown that the residual solvent present in bilayers formed by traditional Mueller-Rudin methods described above considerably modifies the physical properties of the resultant membranes. One solution to this problem is to use long-chain hydrocarbons, such as octadecane or squalene, as solvents [31], as they are more likely to be excluded from the bilayer. White also developed a

technique in which the solvent is "frozen out" of the bilayer by lowering the temperature of black lipid films to below the melting point of the solvent [32,33]. Another approach, proposed by Waldbillig and Szabo [34], produced planar bilayer membranes from a variety of pure lipid mixtures. While these methods eliminate most of the solvent, which is believed to be trapped as microlenses in the bilayer, they are still not able to be used to investigate the effects of membrane asymmetry (i.e., the asymmetrical distribution of lipids and/or proteins), which most natural membranes have shown [35,36,37,38,39].

(b) *Apposition of Monolayers*

Montal and Mueller [40], using a modification of the procedure first described by Langmuir and Waugh [8] and later developed by Takagi, *et al.* [41],were the first to form asymmetrical lipid bilayers that contained a minimal amount of solvent. Experimental apparatus consists of two troughs separated by a septum containing an aperture. A lipid monolayer is applied to the aqueous phase on either side of the septum. When the level of aqueous solution in each compartment is raised above the aperture, a lipid bilayer membrane is formed by the apposition of the hydrocarbon chains from each monolayer.

Lipid bilayers formed from monolayers may not be completely solvent-free, because of the need to use petroleum jelly (vaseline) or silicone grease to coat the membrane support [42,43,44]. When the solvent added with the preconditioning agent evaporates, an oleophilic coat is formed on the support. This improves the conditions for membrane formation, as Teflon is hydrophobic but not lipophilic. Indeed, it was found that the presence of alkane solvent or other non-polar hydrocarbon molecules on the surface of the support, which act as a torus surrounding the bilayer, assists in formation and promotes stability and is a necessary condition for bilayer

formation. Whether the preconditioning agent or non-polar solvent used in the formation is or is not present in the bilayer depends on their water solubility and molecular size relative to the size of the hydrocarbon chain of the lipid [45]. Squalene, a nonsurface-active, long-chain, conjugated hydrocarbon molecule, which is liquid at room temperature, has been used successfully, both as a solvent [31] (as mentioned earlier) and a preconditioning agent [43,46], to stabilise the bilayer. It does not appear to dissolve in the bilayer [31], but instead is partitioned towards the periphery of the membrane and therefore is less likely to modify the lipid bilayer or any other molecules that it may contain [46]. Furthermore, while the purity of petroleum jelly and silicon grease is questionable, squalene has been found to be essentially free of contaminants.

It has been proposed that "solvent-free" membranes are more stable than black lipid membranes; however any comparison between their relative stabilities must involve consideration of the respective areas of each type of membrane. It is known that the lifetime of a membrane is dependent upon its area [47,48], increasing with decreasing area, and "solvent-free" membranes have usually been formed on apertures with smaller diameters.

In this work an adaption of the method used to form bilayers from monolayers was used as will be described in detail in the following Chapter.

(c) *Some Factors Affecting Bilayer Stability*

Determination of the electrical properties, capacitance and resistance, require the application of an electric field across the membrane. The transmembrane potential (or measured potential difference across the membrane) at which the membrane breaks, the breakdown voltage, is dependent upon the past history of the membrane, the duration and nature of the applied potential, the temperature, the lipid composition as well as the

concentration, the concentration of modifiers (or non-lipid compounds), and nature of the electrolyte in the aqueous phase.

Temperature and pH of the aqueous solutions used in any experiment should be controlled or, at least, noted. Temperature is a particularly important variable, as the various structural forms of the lipids forming the liquid crystalline phase, which constitutes the bilayer, are temperature dependent. Formation of BLMs and their stabilities are known to be affected by small temperature changes [15] (although this effect may be less pronounced when the solvent present in the bilayer is kept to a minimum).

Cholesterol is found in cell membranes, occurring at much higher concentrations in the plasma membrane than in many intracellular membranes. As cholesterol exhibits amphipathic character, it is readily accommodated in membranes at phospholipid/cholesterol molar ratios of 1:1 and higher. The polar hydroxyl of the molecule faces the aqueous solvent and the hydrophobic steroid ring is oriented parallel to, and buried in, the hydrocarbon chains of the phospholipids [49]. Many investigations of the distribution of cholesterol and its effects on the lipid bilayers have been undertaken [49,50,51,52,53,54,55].

Addition of cholesterol to phospholipid-containing lipid solutions helps to increase the membrane stability [16,56,57,58], and for this reason it has often been added to membrane-forming solutions in this work to allow a more extensive investigation of each system.

CHAPTER 7 - PREPARATION OF BILAYER LIPID MEMBRANES

Materials used in the preparation and investigation of bilayer lipid membranes are given in Appendix I.

7.1 *BLM-FORMING SOLUTIONS*

An amphipathic molecule (e.g. a phospholipid or oxidised cholesterol) and a neutral lipid solvent are essential constituents of a membrane-forming solution.

Monoolein, PC and PS were supplied as 5 mg of dry compound, while PE was supplied as 5 mg in 0.5 cm^3 of a chloroform/methanol (9:1, v/v) solution. All of these lipids were stored at about -20° C. Natural lecithin was stored as obtained, at about 5° C, in sealed containers. Once it was prepared in n-hexane, the oxidised cholesterol was stored as a solution, also at about 5° C.

BLM-forming solutions were usually prepared fresh each day. Methanol and n-hexane were used as the solvents in the BLM-forming solution; as they are reasonably volatile the time required for them to evaporate from the surface of the solution in the troughs was minimal. Chloroform, being denser, was used only together with one of the two solvents already mentioned. The main solvent used was n-hexane, although it was sometimes necessary to add small amounts of chloroform to ensure that all of the lipids had dissolved. Lipid concentration in the BLM-forming solutions was generally about 1 mM. Unlike the solution necessary for the formation of black lipid films, the final lipid concentration was not required to be at or near saturation.

A Langmuir-Adam surface film balance was used to plot surface isotherms in order to determine the area that is covered by one monolayer with the addition of a certain volume of BLM-forming solution [59]. Three surface isotherms were obtained for each solution and the values obtained were averaged. This procedure was carried out before use of the BLM-forming solutions, so that an estimation could be made of the amount of lipid added to the surface of solutions in the troughs. Concentration of the BLM-forming solutions was usually chosen so that about 20 microlitres of solution corresponded to 10 times the amount of lipid needed to form a monolayer on the surface of the solution in each trough.

7.2 *DESCRIPTION OF THE EXPERIMENTAL SETUP*

The experimental setup was a simplified modification of that used by Tancrède, *et al.* [43]. The system is composed of two Teflon troughs which are placed in a perspex support with a removable cover (Fig. 7.1). A screw is fixed to a perspex bar on one side of the support and is tightened once the apparatus is assembled to ensure that the Teflon film containing the aperture remains positioned securely between the troughs throughout the experiment. The troughs are machined from a solid Teflon block and Figure 7.2 shows the dimensions and also the positions of the film-holders and trough dividers. The dividers separate each trough so that the surfaces of solutions in the main and minor troughs are not connected. As solutions are added to the minor troughs, surface-active contaminants will not be able to pass into the main troughs where the bilayer formation occurs.

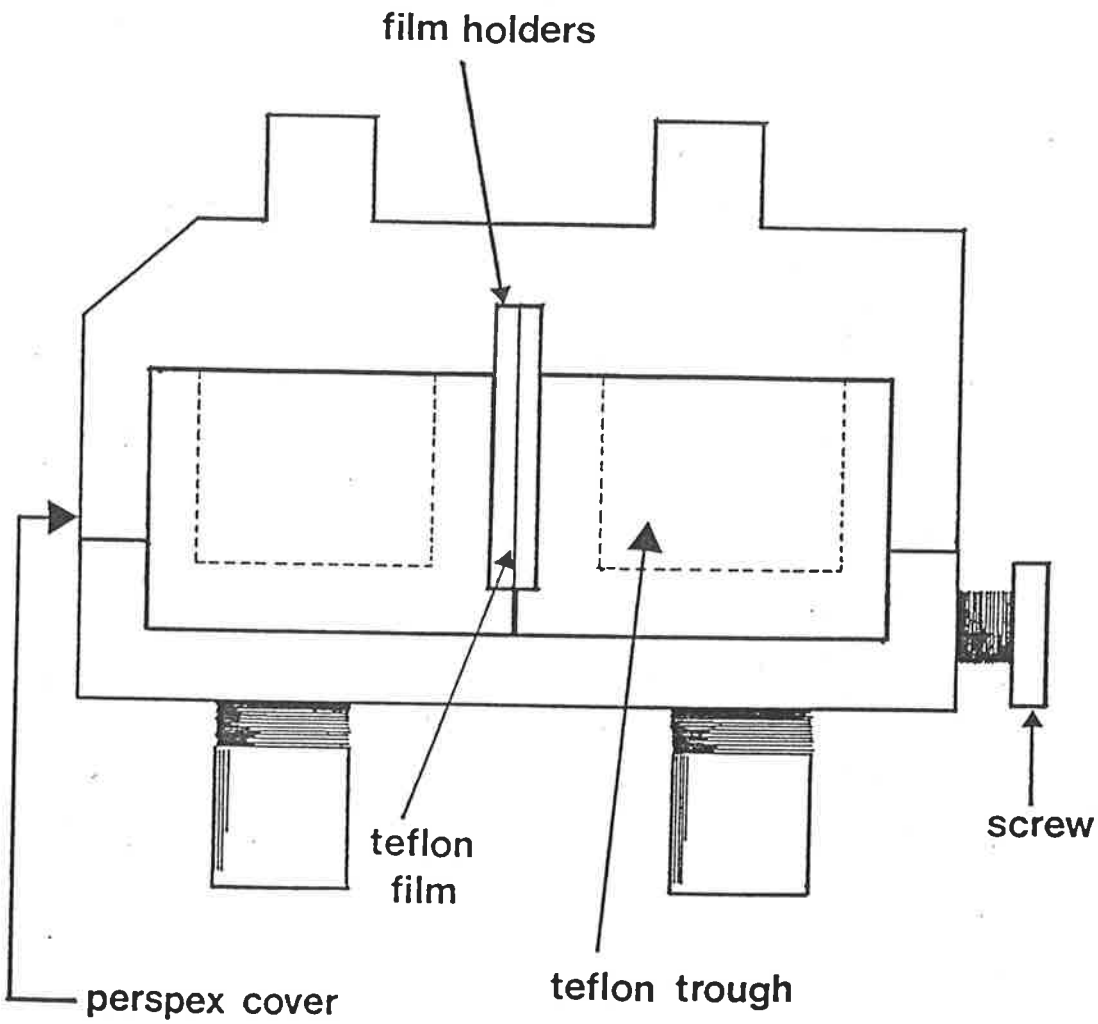


FIGURE 7.1 : Schematic diagram of the apparatus used in the formation of bilayer lipid membranes [Chee Hoong Lai, Honours Report, University of Adelaide, 1987].

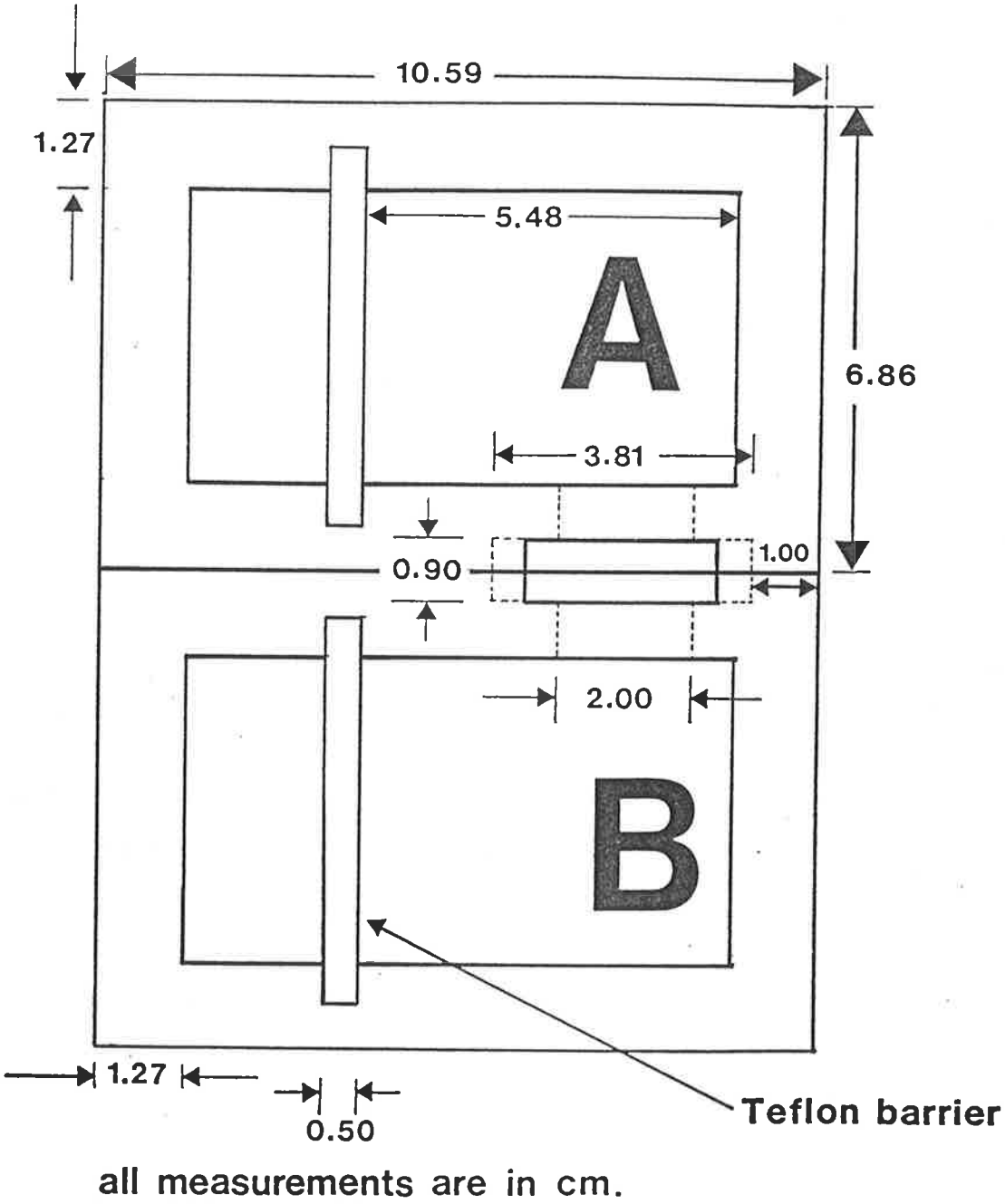


FIGURE 7.2 : Dimensions of troughs and film holders [Chee Hoong Lai, Honours Report, University of Adelaide, 1987].

The thin Teflon film, which is positioned between the film-holders, was the septum used as a support for the lipid bilayers. Lipid bilayers were formed across a small hole in the thin Teflon film (50 mm × 19 μm, supplied by Chemfab Toralon Division). Teflon is a suitable material for both the troughs and the BLM support as it satisfies the requirements of being chemically inert to both aqueous and organic solvents, is non-conducting, and is easily cleaned.

The formation of the aperture is a critical procedure. If the edges are rounded, the monolayers will be unable to pass over the aperture evenly, and it will be difficult to form or stabilise the membranes. An aperture that is too large will also cause the membranes to be unstable; apertures formed from punches with nominal diameters of 0.35 mm, 0.5 mm, or 0.6 mm were used in this work. Actual diameters of the punches were measured with a micrometer and found to be 0.33 mm, 0.50 mm, and 0.55 mm, respectively. Two machine-flattened stainless steel plates 3 mm thick were fixed by a joint or positioning pins [43] (apparatus used is shown in Figure 7.3). A hole of the required diameter was drilled through both plates. The blunt end of the drill was ground smooth and perpendicular to its length so as to give a sharp, circular cutting edge. It was used as a punch to form the aperture in the thin Teflon film, which was clamped between the stainless steel plates. With a firm movement, the drill was brought down through the Teflon film, rotated slightly, and removed. About 6 sheets of Alfoil were also clamped between the plates to support the thin film. The resulting apertures were then inspected under a microscope. Any apertures not suitably circular and smooth were discarded.

The thin Teflon film containing a suitable aperture was trimmed and placed, together with an O-ring, between the two Teflon film-holders with positioning pins, ensuring that the aperture was in the centre of the

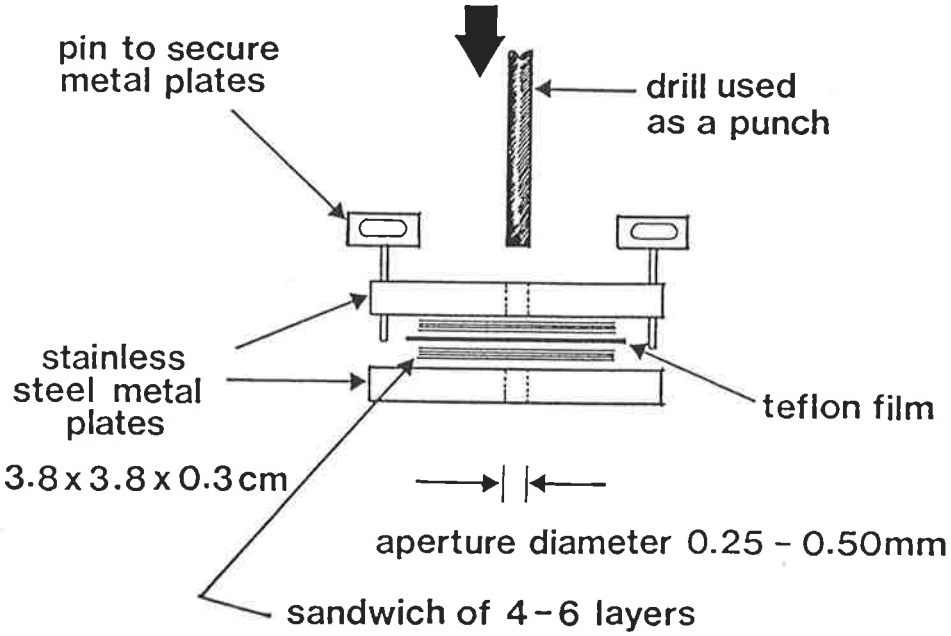


FIGURE 7.3 : Apparatus used to form the aperture in the thin Teflon film [Chee Hoong Lai, Honours Report, University of Adelaide, 1987].

larger hole in the Teflon holders. The diameter of this larger hole decreased gradually from 12 mm to 6 mm across the thickness of each piece. This feature ensures a smooth, uniform flow of the solutions when the levels in the troughs are raised over the aperture where the lipids form the bilayer. The film-holders were then tightly clamped in the cut-away section between the two troughs. Leakage of solution from either of the two troughs was unlikely with the design of this apparatus. Silicone grease (High vacuum grease from Dow Corning, D1400) was used to insulate the two compartments electrically. It was lightly applied between the film-holders and the film, and also to the edges of the film-holders. Therefore an infinite resistance was measured across the troughs when the water levels on the two sides were below the aperture in the thin Teflon film.

The experimental setup described above was placed in a Faraday cage which, when properly earthed, effectively isolates the bilayer from any source of electrical interference. High resistances of the membranes necessitate this isolation of the BLM-forming setup. The Faraday cage rested

on an experimental table which was designed to eliminate, or at least minimise, any vibrations capable of rupturing the membrane. The heavy, three-legged, steel table was laid on three partially inflated rubber inner tubes, which should absorb most of the vibration coming from movement on the floor surrounding the table. Concrete slabs and a slate top on the table added to its weight and helped increase its stability.

All experiments were carried out at room temperature, which was maintained at (22 ± 2)° C.

A Nikon SMZ-2B microscope was used to observe the aperture region when raising and lowering the solutions.

7.3 *CLEANING PROCEDURE*

It is imperative that stringent cleaning rituals are performed before every attempt to form a bilayer is made, as it is accepted that inadequate cleaning of the apparatus can hinder or prevent BLM formation or decrease BLM stability.

Before use, the Teflon blocks and film-holders were soaked in a chloroform/methanol (7:1, v/v) solution [60] for at least 12 hours. The equipment was then rinsed with deionised water, followed by rinsing about 10 times with millipore-filtered water. Between experiments the aperture region and troughs were sometimes cleaned *in situ* by rinsing thoroughly with chloroform and allowing the apparatus to dry at room temperature before further use. When the aperture became deformed or it was suspected that the inability to form stable membranes was due to the cleanliness of the system, the thin Teflon film was discarded and the apparatus was again soaked in the chloroform/methanol solution.

After a suitable aperture had been formed in the thin Teflon film it was soaked in a chloroform/methanol (7:1, v/v) solution. It was then rinsed repeatedly with deionised water and finally with millipore-filtered water.

The rubber O-ring was rinsed in a dilute HCl solution followed by thorough rinsing with millipore-filtered water. No detergent or organic solvent was used in the cleaning of the O-ring.

Glass syringes and plastic tubing used for adding the aqueous solutions to the troughs were rinsed with deionised water after use followed by dilute HCl solution, before finally rinsing with millipore-filtered water.

The Agla syringe used for dispensing the BLM-forming solution was thoroughly rinsed with chloroform.

All other glassware and equipment used in the preparation and storage of solutions were soaked in a detergent (DECON-90) solution, rinsed with deionised water, soaked in dilute HCl solution, rinsed again with deionised water and finally, with millipore-filtered water.

All equipment was allowed to dry, at room temperature, in a dust-free environment, for example, the Teflon apparatus was placed under glass dishes. The millipore-filtered water used throughout was obtained as Milli-Q Reagent Water.

7.4 *PROCEDURE FOR PREPARATION OF BLMs*

During assembly the Teflon equipment was handled using disposable plastic gloves to prevent contamination of the system with surface-active lipids from the hands. It was advisable to wear them for the entire experimental procedure.

As discussed earlier, preconditioning of the aperture region greatly improves both the ease of formation of a bilayer and also its stability.

A 2% (w/w) petroleum jelly in n-hexane solution was tried as the preconditioning agent, but a 2-6% (v/v) squalene in n-hexane solution was found to be more successful, confirming the observations of others [43] and therefore was used throughout this work.

The following procedure was followed for each attempt to form stable BLMs :

i) The thin Teflon film was positioned between the film-holders, which were then clamped between the two troughs (as detailed in the description of the experimental setup). Preconditioning was then performed as the 2-6% (v/v) squalene in n-hexane solution was applied to each side of the film and film-holders. Two or three applications of squalene solution were made using a thin paint brush. The setup was left to dry at room temperature for at least 1 hour.

ii) The perspex holder containing the troughs was placed inside the Faraday cage and the light source (FSE Scientific, 6 volt/20 watt) positioned behind the apparatus so as to illuminate the aperture region. During monitoring of the stability and properties of membranes, the light source was often used for several hours. The temperature of the bathing solutions was raised by

about 2° C after 4 to 5 hours of illumination of the aperture. Whether or not the light was on affected neither stability nor properties once the membrane was formed.

iii) Both troughs were then filled with the buffer/electrolyte solution so that the meniscus of each solution was just touching the thin Teflon film but still well below the aperture. Two stainless steel capillaries were fitted to the ends of the plastic tubing of the syringes and small holes in the perspex cover over the minor trough accommodated these capillaries during raising and lowering of the solutions. Nitrogen was usually bubbled through the solutions in the minor troughs for several minutes, before the tubing delivering it was raised above the surface to allow continuous nitrogen flushing of the system throughout the experiment. Gas bubbles, which are likely to cause optical and electrical interference or cause rupture of the membrane [15], were avoided and removed when found in the main trough.

iv) The surface of solution in both troughs was swept clean with thin tissue paper (Olympic Lens Cleaning Tissue). Tweezers were used in order to avoid touching the paper with fingers. The process was repeated at least 6 times, discarding the paper after each sweep.

v) Using an Agla microsyringe the BLM-forming solution, consisting of the lipid(s) in an organic solvent, with or without the addition of a membrane modifier, was delivered to both troughs. Each trough contained a solution with a surface area of 22.5 cm^2. The microsyringe was held very close to the nitrogen/aqueous interface before being adjusted to give a small drop on the end. This drop was then touched against the surface of the solution. It was applied close to the aperture so that any surface impurities would be swept back away from the aperture [14]. The amount of lipid solution added was often in large excess compared with the amount needed to form a monolayer, as the stability of membranes seemed to be enhanced with the

spreading of lipid films instead of just a monolayer [43]. Regular observation of the levels of the solutions in both troughs and adjustments using the syringes ensured that they remained below the aperture during application of the BLM-forming solution.

vi) After the monolayers or lipid films were spread, sufficient time (about 10 to 20 minutes) was allowed for solvent evaporation to occur.

vii) To form a bilayer two monolayers were brought into contact in the aperture. This was achieved by raising the aqueous solutions above the aperture. Adjustment of the level of the solutions was always performed using the glass syringes, with the attached metal and plastic capillaries, to add the solution to the minor troughs. One monolayer was adjoined to the Teflon film and then the second one. Usually the solution in the back trough was raised first, to facilitate viewing of the water levels with the microscope. It was considered beneficial [61] to initially raise and lower both sides separately so that the aperture region was also "preconditioned" with the lipid monolayer. The syringes were sometimes used in conjunction with a mechanical device, which allowed the solutions to be raised smoothly at several different rates. Generally, a very slow rate was used. However, careful manual adjustment of the level of the solutions resulted in the formation of stable membranes, so use of the device was considered to be time-consuming and unnecessary.

viii) If a bilayer was formed the solutions in both troughs were raised well above (i.e. at least 2 mm above) the aperture and made level. When the solution levels are equalised the membrane is planar and not otherwise deformed by a difference in hydrostatic pressure between the two sides [43].

CHAPTER 8 - ELECTRICAL MEASUREMENTS

The cell arrangement for the electrical properties of BLM is:

non-polarisable electrode.	aqueous solution 1	BLM on Teflon support	aqueous solution 2	non-polarisable electrode

Aqueous solutions serve as ohmic contacts to the BLM, which can be represented by an equivalent circuit consisting of a capacitor in parallel with a resistor.

Saturated calomel electrodes were used as the non-polarisable electrodes in this work and a platinum wire was the third electrode when required. Reversible saturated calomel electrodes did not introduce a potential difference of greater than 2 mV into the circuit.

The high resistances of unmodified BLM necessitated that great care was taken in the insulation of switches and connections to avoid current leakage.

After formation of the membrane, application of an intermittent voltage pulse allowed the electrical properties to be monitored. It has been suggested that the stability of the membrane is improved if the voltage applied is kept below 30 mV and is intermittent rather than continuous [14]. The following sections outline the methods used to determine the capacitance and resistance of the membranes.

8.1 *CAPACITANCE*

Determination of the capacitance of a membrane involved using a Universal Programmer (Model 175, Princeton Applied Research, Princeton, N. J.) to apply a single pulse of amplitude 25 mV (sometimes 50 mV or 100 mV) and pulse width 1 msec across the membrane. Current flowing through the membrane was determined by measuring the voltage drop across a resistor (10^5 Ω) in series with the membrane, recording the signal on a cathode ray oscilloscope (Model 434, Tektronix, Inc., Beaverton, Oregon, U. S. A.) and also feeding it into a transient recorder (Model DL905, Datalab). The transient recorder was programmed to take 1024 samples of the current signal at varying rates, with a resolution of 8 bits. A computer was used on-line to collect the data and analyse the response to the charging of the series RC circuit. Data collected with the membrane potential at zero were subtracted from those values found after the small pulse was applied. This zero correction was carried out before further analysis of the data.

A voltage follower was added to the circuit between the electrodes and the transient recorder, as it effectively isolates the signal and measurement circuits by presenting a very high impedance to the signal and a very low impedance to the load.

The series RC circuit acted as a high-pass filter (Fig. 8.1), where the output was taken across the known resistor [62].

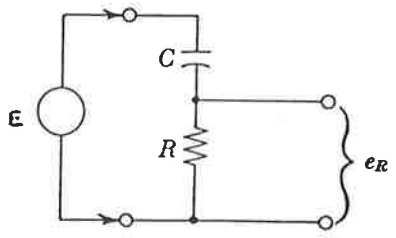


FIGURE 8.1 : A high-pass filter [62].

After a single pulse, E, (usually 25 mV) has been applied to the circuit the capacitor begins to charge to the applied potential. During the charging process $e_R = i\,R$ and $e_C = q/C$, where i is the current, R is the resistance, q is the charge on the capacitor, and C is the capacitance. From Kirchoff's law:

$$E = i\,R + \frac{q}{C} \qquad (1)$$

As the charge on the capacitor increases, i decreases and the rate of charging is decreased. Substitution of dQ/dt for i in Eqn. (1) and integration gives the following expression for e_C ,

$$e_C = E(1-e^{-t/RC}) \qquad (2)$$

which shows that $e_C = 0$ when $t = 0$ and e_C approaches E exponentially as t approaches infinity. The product $RC=\tau$ has units of seconds and is known as the time constant.

From Eqns. (1) and (2), the following is obtained:

$$e_R = Ee^{-t/RC} \qquad (3)$$

When t = 0, all of the applied potential, E, has developed across the resistor. Current and e_R then decreases with time and are zero when the capacitor is fully charged. The plot of e_R vs. time is shown in Figure 8.2. After one time constant the current and hence e_R has dropped to e^{-1} of the value of the applied potential.

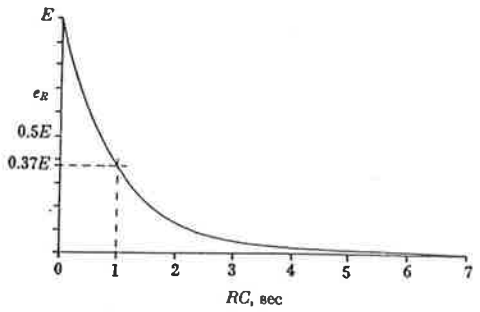


FIGURE 8.2 : e_R during series RC charging [62].

The computer program used in the analysis of the data calculated the capacitance of the membrane using two different methods:

i) *ln analysis* : From Eqn. (3) ,

$$\ln e_R = \ln E - (\frac{t}{RC}) \qquad (4)$$

where e_R is the voltage across the resistor. Computer analysis yielded an intercept of ln E and a slope of -1 / RC [4] after consideration of ln e_R vs. t .

ii) *Integral analysis* [27,40]:

$$\text{Charge, } Q = CE \qquad (5)$$

As $I = \frac{dQ}{dt}$,

$$C = \int I\, dt / E \qquad (6)$$

Also, $I = e_R / R$, where $e_R = Ee^{-t/RC}$.

Therefore,

$$C = \int Ee^{-t/RC}\, dt / RE \qquad (7)$$

Computer analysis determined the integral, $\int Ee^{-t/RC}\, dt$ from the plot of e_R vs.t , by calculating the area under the curve. Two methods, involving either Newton-Cotes' rule or Simpson's rule, were used with the value found being exactly the same for each case.

Capacitance values determined from both the *ln* and *integral* analyses were usually in agreement. Note that the value of E calculated from the intercept was consistently lower than the actual voltage applied. During calibration of the system the capacitance values determined using the voltage calculated from the intercept was considered to be slightly more accurate than when the value of the actual applied voltage was used.

Measurements of capacitance were also performed with a Universal Bridge B641 (frequency = 10^4 rad sec^{-1}), from the Wayne Kerr Company Limited, New Malden, Surrey. A low frequency AC bridge signal [63] was used to measure the equivalent parallel capacitance and conductance of the circuit [64].

Capacitance values for each system were obtained by subtracting the capacitance resulting when the solutions in both troughs were below the aperture (ie. the capacitance of the septum) from the total capacitance found with the solutions above the aperture with a membrane in place. This procedure was used in all capacitance measurements irrespective of the method used.

The system was calibrated with capacitors of known values and the accuracy found to be better than 2% for both the DC and Wayne-Kerr bridge methods. Signal averaging was not performed for either the DC or AC method, but at least 5 measurements were taken for each membrane and the capacitance values obtained were averaged.

Capacitances of the electrodes are large but, since they are in series with the bilayer, their effect on the measured capacitance is small.

Shielded, low-noise cables were used between the instruments to minimise the effect of stray capacitance. Stray capacitances of less than 100 pF found in the circuit used for these measurements were probably due principally to the voltage follower and cancelled as the capacitance of the system with solutions in both troughs below the aperture was subtracted from the total capacitance.

8.2 *RESISTANCE*

Resistance was measured by a DC method which involved applying a small voltage (0-100 mV) across both the BLM and a known resistance, R_i (10^5-10^{10} Ω), in series with the membrane, via a pair of reversible electrodes. The voltage drop across the BLM was measured using a high impedance electrometer (Model 602, Keithley Instruments, Inc., Cleveland, Ohio) and BLM resistance found by the direct application of Ohm's Law :

$$E = i\ R \qquad (8)$$

where E is the voltage, i the current and R is the resistance.

$$\text{Membrane resistance, } R_m = \left[\frac{E_m}{(E_i - E_m)} \right] R_s \qquad (9)$$

where R_S is known resistance of the resistor in series with the membrane,

E_i is known input voltage, and

E_m, the membrane potential, is the voltage appearing across BLM. The voltage usually applied was 25 mV, which was low enough so as not to introduce any instability into the membrane.

It can be seen that the ratio of resistances is equal to the ratio of the respective potential drops. The most accurate values of membrane resistance, R_m, are found when the known resistance is almost that of the membrane ($R_i \sim R_m$), so that $E_m \sim 0.5\ E_i$.

BLM resistance was also determined from the plot of i against E, using the X-Y recorder incorporated in the setup used for obtaining cyclic voltammograms. As I = E / R, the slope of the plot allows the resistance to be determined. The resistance of an unmodified membrane is ohmic until the breakdown voltage is applied.

Specific resistance values are expressed as Ω cm^2 [65], requiring the measured resistance to be multiplied by the area of the membrane. Specific capacitance values are expressed as nF cm^{-2}, obtained by dividing the capacitance measured by the area of the membrane.

In practice, the determination of the membrane area is the limiting factor in obtaining accurate and, more importantly, reproducible specific electrical values. Therefore, although greater accuracy and precision for electrical measurements was possible with modifications to the setup, this was not considered necessary due to the uncertainties in the estimation of the membrane area. For all calculations the membrane area was taken to be equal to the geometric area of the aperture. Accurate measurements of the aperture diameter were performed with an OMT (Optical Measuring Tools, England) measuring microscope with projection apparatus and graticule. Diameters of the apertures formed from 0.55 mm, 0.50 mm, and 0.33 mm drill punches were determined to be 0.52 mm, 0.47 mm and 0.31 mm, respectively. A standard deviation of less than 1% was observed for about 20 samples of each size. These measured values were used in any calculations where the area of the membrane was required.

CHAPTER 9 - DISCUSSION OF THE PROPERTIES OF BLMs

Although the main purpose of this work was to investigate the electron transfer occurring across these model systems, it was first necessary to determine some of the properties of the bilayers involved in the measurements. Controversy has always existed over which planar bilayers are the best models for biological membranes, but all researchers have agreed that lipid bilayers are not well behaved or easily understood. An important consideration to be addressed here,which is of much relevance when considering model systems for biological membranes, is whether or not the properties of bilayers formed by apposition of monolayers are less likely to be affected by the presence of solvent, than those formed by the more traditional Mueller-Rudin-Tien method. Our attempt to answer this question has been via comparisons of some of the static electrical properties of the bilayers.

(a) *Formation and Stability*

In general, electrical measurements are made on a system that is much larger than the bilayer of interest, comprising the transition zone between the bilayer and the solid support (known as the torus, annulus or Plateau-Gibbs border), the Teflon support, the troughs, the aqueous solutions on either side of the membrane and the electrodes [66]. White, *et al.* [45] have shown that a transition zone is present in membranes prepared from monolayers and its presence is, in fact, necessary for the formation of stable membranes; clearly, even if it is of less significance than that present in black lipid films, it still influences the electrical properties of membranes [66]. In this context, a membrane is generally taken to mean the structure

covering the entire aperture, including the torus, while bilayer refers to the regions of the membrane that are only two molecules thick [66,67].

Experimental factors contributing to the lack of success in obtaining membranes have been listed by earlier researchers and many of them, such as the presence of impurities in the system, inadequate preconditioning, and the use of unsuitable BLM-forming solutions, were encountered in this work, preventing the formation of stable membranes. Successful experiments became more routine with time, although strict adherence to the careful procedures for preparing solutions and apparatus was always necessary.

In agreement with others, it was found that the membranes were most prone to rupture during the first few seconds after their formation; some membranes were stable for up to several hours.

From Langmuir trough measurements of each BLM-forming solution, an estimate of the amount of surface-active material added to the surface of the solution in each trough was made. The amount of lipid added was sufficient to form from about 5 to over 100 monolayers, with the majority of membranes formed from between 10 and 30 monolayers on the surface of the solutions. Benz, *et al.*, [27] also added much more of the lipid to the surface of the solution in both troughs than was needed to form a monolayer. Membranes were, therefore, formed from multilayers rather than monolayers, although it is only a monolayer from each trough that covers the aperture when the solutions are raised above the aperture. Tancrède, *et al.* [43] used troughs that were specially designed to allow formation of bilayers from a single monolayer on the surface of solution in each trough, also permitting characterisation of the monolayers before formation. Even with this adapted apparatus, they found that it was slightly more difficult to form a membrane from monolayers rather than lipid

films. In this work, addition of excess BLM-forming solution to the surface of the electrolyte was often required to obtain stable bilayers, but no noticeable differences were observed in the properties of the membranes formed from many or few monolayers. It should be noted that the terms *several monolayers*, *multilayers*, and *lipid films* are not meant to suggest that the lipids are stacked uniformly on the surface of the aqueous phase, as clumps or reservoirs of lipid are perhaps more likely when an excess is added.

(b) *Membrane Capacitance*

The formation of membranes was usually followed by observing the continuous capacitance curve on the oscilloscope. The capacitance increased above the background level of the thin Teflon film as the second solution was slowly raised above the aperture. Thinning of the bilayer occurs with the loss of any solvent present causing the capacitance to increase. If the capacitance of a newly-formed bilayer was very low or increased very slowly to its limiting value and did not stabilise within 10 minutes, it was thought that it contained a considerable amount of residual solvent. If, on the other hand, the bilayer was considered to be relatively solvent-free, a steady value was attained within a few seconds [68]. The most commonly used solvent, n-hexane, having limited solubility in water, would tend to diffuse into the aqueous phase and escape into the atmosphere. The presence of chloroform, in particular, has been known to cause long-term drifts in the capacitance values [64]. Unusually low values of specific capacitance can also result if there is water present in the BLM-forming solution which causes the films to be abnormally thick or have thick patches. Generally, for unmodified membranes, neither capacitance nor resistance values varied significantly with time after both solutions were level above the aperture. The average capacitance values given in Table 9.1 exclude values which were extremely low or which varied with time.

BLM-FORMING SOLUTION	NO. OF BLMS	CAPACITANCE ($nFcm^{-2}$) [calculated using ln analysis]	CAPACITANCE ($nFcm^{-2}$) [calculated using integral analysis, with intercept voltage]
Monoolein	88	869 ± 11	852 ± 25
Monoolein/cholesterol	20	874 ± 8	867 ± 8
Monoolein/TCNQ	120	975 ± 55	981 ± 12
Monoolein/cholesterol / TCNQ	15	913 ± 6	908 ± 3
	75	732 ± 16	727 ± 5
Natural lecithin	11	659 ± 2	658 ± 4
Natural lecithin/cholesterol	9	741 ± 2	730 ± 4
Natural lecithin/TCNQ	6	701 ± 3	702 ± 1
Natural lecithin/I_2	8	717 ± 3	713 ± 1
Natural lecithin/ox. chol./TCNQ	5	544 ± 1	561 ± 1
Natural lecithin/ox. chol./I_2	26	725 ± 4	724 ± 8
PE	7	760 ± 2	763 ± 3
PE/TCNQ	97	676 ± 3	678 ± 6
PE/PS symmetrical	27	754 ± 13	765 ± 9
PE/PS/TCNQ symmetrical	52	819 ± 10	821 ± 2
PE/PS/I_2 symmetrical	2	666 ± 1	636 ± 1
PS	4	812 ± 4	792 ± 1
PS/monoolein			

TABLE 9.1 : Capacitance values (standard deviations given) for membranes formed on 19 μm thick Teflon film in a bathing solution with ionic strength of about 0.1 M.

Often when the solution in one of the troughs had been raised the bilayer seemed to be formed and the maximum capacitance value was reached even before the aperture was completely covered by the solution on the other side. This phenomenon seems to suggest that the lipid molecules 'run' ahead of the solution and momentarily form a bilayer supported by a solution on only one side. Visual observation of the aperture area distinguishes the above behaviour from that also observed in this work, and described earlier by other researchers [40], where the water level rises rapidly above the aperture as soon as the level of the solution being raised reaches the lower rim of the aperture. The latter action is believed to be due to capillarity. In experiments where preconditioning of the Teflon film-holders and thin Teflon film with the squalene solution was not performed, this capillary action was enhanced and, in all cases, caused the rupture of the bilayer during formation. This reinforces the observations of other researchers that preconditioning is necessary for not only the stability of the membrane, but also for its formation.

Several investigators have demonstrated that solvent-containing lipid bilayers show voltage-dependent capacitance. Babakov, *et al.* [69] were the first to note that capacitance increases with the square of the applied voltage, and later White [70] performed an extensive study of the effect. The voltage dependence is believed to be due to the geometric changes brought about by electrocompression which results in the decrease of membrane thickness as the hydrocarbon solvent is squeezed out into microlenses, with the torus kept at approximately constant area. Voltages of 25 mV, 50 mV, and 100 mV were successively applied to some of the membranes formed and there was no change in the capacitance with voltage within the limits of experimental error. The membranes used in this work are therefore determined to be "solvent-free" using the criteria introduced by earlier researchers [27,31,69]. However, Alvarez and Latorre [71] showed that

bilayers, formed by the apposition of two separate monolayers spread at the air-solution interface, also exhibited increased capacitance as a linear function of the square of the membrane potential. It should however be noted that the changes observed are much faster and 3 orders of magnitude smaller, than those observed in black lipid films reflecting the relative amounts of solvent present. In their study, the method of determining the capacitance used was sufficiently sensitive to observe the very small changes occurring.

Capacitance values for the various BLM-forming solutions are given in Table 9.1. The standard deviation for membrane capacitance for the majority of the systems investigated was less than 1%.

Statistical evaluation of these data using 'tests of significance' [72] (Appendix II) showed that the capacitance values obtained were statistically different for some lipid systems.

Presence of TCNQ in the BLM-forming solutions seemed to increase the capacitance of resulting systems. Addition of another membrane modifier, I_2 , decreased the capacitance of natural lecithin and natural lecithin/cholesterol membranes, but increased the capacitance of symmetrical PE/PS membranes.

(c) *Membrane Resistance*

Compared with values for black lipid membranes, and also other membranes formed from monolayers [40], membrane resistance was found to be slightly lower at 10^5-10^7 Ω cm^2. BLM resistance is noted for being irreproducible from one membrane to another, although the resistance of a given membrane was usually found to be relatively constant until just before it burst [Fig. 9.2].

BLM-FORMING SOLUTION	RESISTANCE (Ωcm^2)
monoolein	[1] $(2.0 \pm 0.6) \times 10^6$ [10] [2] $(1.0 \pm 0.6) \times 10^5$ [3]
PE / PS	$(5.0 \pm 0.5) \times 10^6$ [8]
monoolein / TCNQ (no redox couples)	$(2.5 \pm 1.0) \times 10^5$ [6] $(4.5 \pm 1.5) \times 10^5$ [6] $(2.0 \pm 1.0) \times 10^6$ [4]
monoolein + cholesterol / TCNQ (redox couples present)	$(7.8 \pm 1.3) \times 10^4$ [4]

TABLE 9.2 : Resistance values for some membranes. Values in square brackets are the number of membranes formed.

[1] Electrolyte solution is 0.1 M NaCl.

[2] Electrolyte solution is 0.1 M CH_3COOH / 0.1 M KCl.

It has been noted that the appearance of lipid "crystals" at the periphery of BLMs lowers the resistance by an order of magnitude or so, presumably by providing low resistance pathways [73]. Resistance values of membranes containing visible "crystals" were rejected.

(d) *Reproducibility of Electrical Properties and their Precision*

BLM capacitance was generally found to be more reproducible from one membrane to the next than resistance, but the average values for the same systems measured on different days, or more specifically with different setups, showed a much greater variation. Recently, Brullemans and Tancrède [66] have suggested that completely reproducible conditions for

producing membranes from monolayers are unlikely. There is a significant dependence on the thickness of the thin Teflon film supporting the aperture ; the thickness of the Teflon film across which the bilayers are formed is thought to affect the volume of the transition region and possibly, also its composition. It is not possible that punching of the apertures or preconditioning with the squalene solution can be repeated precisely enough to produce membranes of exactly the same dimensions (ie. with the same torus). Almost all the values quoted here were obtained using a different setup and therefore a different aperture and different preconditioning conditions.

Both the formation and measurement of the aperture was considered to be sufficiently precise, with standard deviations of about 1% observed for the diameter sizes of the apertures formed from the three punches used. When comparing different systems it is sufficient that the precision is high, but for comparison of measurements made by different researchers the accuracy must also be considered.

The accuracy of the estimation of the area also limits the reproducibility of the measured values. For calculation of specific values of capacitance and resistance, the area of the bilayer was assumed to be equal to the area of the aperture. This probably results in an overestimate of the actual area, since there must be a torus surrounding the bilayer that occupies part of the area of the aperture which, because it is many orders of magnitude thicker, makes no direct contribution to the electrical properties [18,45], although its presence does have an effect. Thus it is possible that the values of capacitance quoted from this work may be slightly lower and resistance values slightly higher than values obtained under exactly the same experimental conditions, but using more sophisticated methods for determining the actual area of the bilayer [14,70].

The thin transparent Teflon film, which is used as a support for the aperture, allowed precise adjustment of the water levels. As the meniscus levels of the solutions were made level after both solutions were raised above the aperture, the capacitance and resistance values presented are those corresponding to a membrane across which the difference in hydrostatic pressure is assumed to be negligible. Therefore, there should not be any significant bowing of the bilayer, which would increase its area and introduce an error into the calculation of specific capacitance and resistance values. Benz, *et al.* [27] also observed that the capacitance of the membrane is at a minimum when the levels of solutions in both troughs are equal.

(e) *Results and Comparison with previous data*

Capacitance values for several systems are presented for comparison with the values found in this work for similar systems (Appendix III). Differences between the capacitance value for each lipid system are less than 20%, but consideration must be made of the experimental conditions. In cases where incomplete information is given about the method and conditions of formation, it is not possible to make meaningful comparisons.

The breakdown voltage was determined experimentally by gradually increasing the applied voltage until the BLM ruptures. Rupture was observed on the oscilloscope by the disappearance of the capacitance curve. Membranes formed in this work generally burst after a potential of at least 200 mV had been applied across them. The breakdown potential of membranes tends to occur at voltages between 150 and 500 mV, but for most lipids it is around 300 mV [14]. Solutions of less than pH 4 or greater than pH 8, exhibit lower breakdown potentials [74].

The presence of cholesterol is known to have an "ordering" effect on the membrane, decreasing membrane permeability and increasing stability. Membrane thickness is also altered with the addition of cholesterol to other lipids in the BLM-forming solutions. Incorporation of a cholesterol molecule, oriented normally to an interface with its hydroxyl group in the aqueous phase, and being approximately equivalent in length to a normal alkyl chain of 14 carbon atoms, will cause the membrane, composed of lecithin (or another lipid) with average chain length of 18 carbon atoms, to become thinner [74, 75]. The following equation is true if it is considered [73] that a bilayer behaves like a parallel plate capacitor.

$$\text{Membrane capacitance, } C_m = \frac{\varepsilon_m}{4\ \pi\ t}$$

where ε_m is the dielectric constant, and

t is the membrane thickness.

The observed decrease in thickness is therefore sufficient to cause a slight increase in capacitance but it was also discovered that the presence of cholesterol increases the dielectric constant of the hydrocarbon chains [57] , and it was this that contributed more to the increased capacitance value. The dielectric constant of the hydrocarbon residues of cholesterol has been estimated to be 2.39, compared to 2.20 for monoolein (18:1) [23].

Therefore, with BLM-forming solutions consisting of lipids having hydrocarbon chain lengths of 18 carbon atoms, the addition of cholesterol is expected to increase the capacitance of the membrane. Hanai, *et al.* [57] observed an increase in membrane capacitance when the mole ratio of cholesterol/lecithin was at least 0.8, with capacitance gradually increasing as more cholesterol is added to the BLM-forming solution until at higher mole ratios the capacitance stabilised and no further changes were observed. Ohki [74] observed a similar effect when PC and cholesterol

constituted the BLM-forming solution, but it was found that the capacitance increased as the concentrations of cholesterol in the sample solution increased *until* the concentration reached 80% in weight, with increasing concentrations having no further effect.

When cholesterol was added to BLM-forming solutions in this work, the resulting capacitance values were consistently and statistically lower than those values formed from solutions not containing cholesterol. This was true for all the systems studied with the exception of monoolein, where a slight (that is, less than 1%) increase was found for membrane capacitance when cholesterol was present.

Black lipid films are usually employed in studies of the effects of cholesterol on the lipids of bilayers, therefore the lack of solvent could contribute to the discrepancy between the previously reported effects and those observed in this work.

It is possible that the relatively inaccurate determination of the area contributed to the difference observed between capacitance values in this work compared to those found by other researchers. This should not be considered a major reason as the method used here to calculate the area is widely used.

Comparison with natural membranes : Electrical measurements on natural membranes result in resistance values ranging from 10^2 to 10^5 Ω cm^2 [76] and capacitance values in the range 0.5 to 1.3 $\mu F\ cm^{-2}$ [77]. Biological membranes, unlike the bilayers used in this work, have translocators that modify the membranes and lower their resistance. The obvious difference between artificial films and natural bimolecular membranes is the presence of solvent hydrocarbon in the former. Langmuir trough experiments of earlier researchers [78] showed that it is possible that cell membrane lipids are less closely packed than the chains in artificial

films. Thus, the hydrocarbon solvent may act as a filler of space which would otherwise be occupied by more polar molecules, such as water. If this were true, it is expected that the capacitance and conductance of the artificial films is smaller than the corresponding property of a membrane containing no solvent [64]. Average capacitance values for black lipid films are generally around 450 nF cm^{-2}. Solvent-containing membranes, being thicker, have lower capacitances than solvent-free systems [68].Therefore the relatively high specific capacitance values (up to 900 nF cm^{-2}) found for membranes used in this work (Table 9.1) confirm that while the membranes may contain slight amounts of solvent it is certainly much less than that in black lipid films.

It should be noted that the systems presented here are suitable for investigations of the role of asymmetric lipid-lipid and protein-lipid distributions and interactions in membranes.

Different values for resistance and capacitance of membranes have been found by different researchers, even when the BLM-forming solutions are the same. When comparing observed properties of model membrane systems, detailed information on all the experimental conditions, the thickness of the supporting septum, and the number of membranes formed must be given.

When a membrane system is being considered as a model for biological membranes it is important that each membrane is stable and its properties are shown to be unvarying over reasonable periods of time and reproducible. It can not be assumed that every lipid bilayer system will be homogeneous and at equilibrium [25]. The systems studied were found to be reasonable models for cell membranes and were therefore used in studies on electron transfer.

CHAPTER 10 - ELECTRON TRANSFER

10.1 *INTRODUCTION*

As early as 1928, Lund [79] suggested that electrical potentials in cells were the result of redox reactions occurring on either side of the cell membrane. In support of the electronic, rather than exclusively ionic, behaviour of living systems, Szent-Gyorgyi suggested that energy in living systems might be transferred by conduction bands [80]. The transduction of solar energy into chemical energy by green plant photosynthesis occurring in the thylakoid membrane [81] and oxidative phosphorylation in cell respiration, which takes place in the cristae membrane of mitochondria [82], are just two examples of electronic processes in biological membranes.

It has been suggested that the mechanism of electron transfer in living systems is electrostenolysis, which was first observed by Becquerel [83] and Braun [84]. Electrostenolysis describes the phenomenon of coupled electrochemical reactions occurring on both sides of the membrane when a direct current is passed through a membrane (or barrier) of high electrical resistance separating two aqueous solutions. Oxidation takes place on the side facing the negative electrode and electrons move across the membrane.

Electron-transfer systems of biomembranes usually consist of protein moieties, which are imbedded in the matrix of lipids forming the bilayer. Mueller, *et al.* [11,85] were the first to demonstrate that the addition of excitability inducing material (EIM), such as proteins, reduced the resistance of phospholipid bilayers and induced characteristics similar to nerve membranes.

An unmodified bilayer membrane behaves essentially as an insulator, exhibiting a high resistance (DC) and low permeability for ions, but when certain compounds are incorporated into such systems, they become electron-conducting. Substances interacting with the lipid membrane (by adsorbing on the surface or penetrating the membrane) and changing its electrochemical characteristics are called modifiers. BLM modifiers may be divided into five categories:

i) those altering the passive electrical properties,
ii) those changing the mechanical properties,
iii) those conferring ion selectivity,
iv) those inducing electrical excitability, and
v) those generating photoelectric effects [15].

Compounds such as antibiotics, drugs, poisons, dyes, and detergents, which in many cases affect the membranes of living cells, also drastically alter the electrical characteristics, structure and mechanical properties of model lipid bilayers.

When pigments are incorporated into BLMs they become photoelectric, so that light-induced electron-transfer and redox reactions have been observed [86,87,88,89,90,91,92] and recently reviewed [93]. Evidence for electronic processes occurring in BLMs in the absence of light has more recently been investigated by Tien, *et al.* [94,95,96] , with the black lipid films behaving as bipolar redox electrodes. Contrary to the earlier belief that current carriers involved in the dark conductivity in unmodified BLM are most likely ions, rather than electrons and holes, the results indicate that electron movement is occurring across the lipid bilayer and redox reactions are occurring at the membrane/solution interfaces.

In this work the electrical properties of unmodified and modified BLM were investigated by cyclic voltammetry. Tien and co-workers have

explored this area of research using black lipid films, which contain considerably more solvent than the membranes used in this work. The following details the study of the behaviour and properties of those membranes containing little or no solvent, as it is, after all, not a natural component of biological membranes.

10.2 *THEORY AND EXPERIMENTAL*

As recognised by many other researchers the BLM system is an ideal model system as it allows easy access to both sides of the membrane. Voltammetric (or current-voltage) techniques are easily performed with such a setup. Cyclic voltammetry is very useful for investigating redox reactions in bilayers. Conventional voltammetry and, more specifically, cyclic voltammetry are mentioned below before the discussion of their application in the investigation of BLMs.

10.2.1 VOLTAMMETRIC TECHNIQUES

[97,98,99,100,101,102,103,104]

Voltammetry is the measurement of the current which flows at an electrode as a function of the potential applied to the electrode. Usually, the electrochemical cell for these measurements consists of three electrodes, referred to as working, reference and counter (or auxiliary) electrodes. Under certain conditions a two-electrode system is suitable. In the voltammetric experiment (Fig. 10.1), the potential of the working electrode is controlled by the potentiostat. The purpose of having a counter electrode is to avoid large currents being passed through the reference electrode during the potential scan;large currents are capable of changing the potential of the reference electrode. Attention is focussed on the working electrode, but the current at the counter electrode is equal, but of opposite sign, to that at the working electrode. The current flowing through the cell (between the working electrode and the counter electrode) is then plotted as a function of

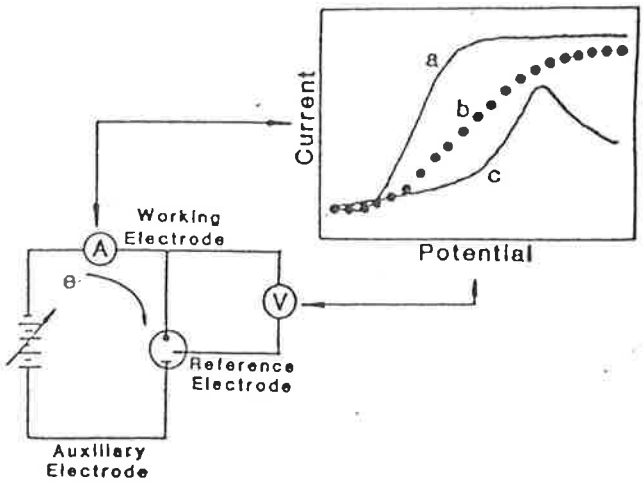


FIGURE 10.1 : The Voltammetric Experiment. Current passing through the working electrode is measured as a function of the applied potential. The curves represent samples of various kinds of voltammetric techniques [103].

the working electrode potential. This potential is the voltage difference between the working electrode and an independent non-polarisable reference electrode. The device used to measure this potential difference should have a high input impedance so that negligible current is drawn through the reference electrode,ensuring that its potential remains constant. Current-voltage (*i* vs. E) response patterns, obtained during the potential scan by measuring the current at the working electrode, are known as voltammograms. The type of voltammogram observed depends on the way that the power supply is varied and on the physical and electrochemical properties of the cell. The faradaic current that flows at any time is a measure of the rate of the electrochemical reaction taking place at the electrode. It is largely dependent on the following factors:

i) the rate at which the species moves from the bulk solution to the electrode (mass transport), and

ii) the rate at which electrons transfer from the electrode to the species in solution and *vice versa* (charge transfer).

Chemical reactions preceding or following the electron transfer and additional surface reactions, such as, adsorption or desorption, also affect the electrode reaction rate, but will not be considered here.

The modes of mass transport are :

i) diffusion - movement of a species under the influence of a concentration gradient,

ii) convection - stirring, using mechanical vibrations of the cell or movement caused by temperature or density gradients, and

iii) migration - movement of a charged body under the influence of a gradient of electrical potential.

Keeping the solution quiescent and adding a supporting electrolyte eliminates the convective and migrational modes of transport. If the current is controlled by mass transport or a steady state process, it may be determined from the flux associated with the change in concentration given a change in distance from the electrode:

$$i\ (t) = n\,\mathrm{FAD}\,\frac{\partial C(x,t)}{\partial x}\ \Bigg|_{x=0} \qquad (1)$$

where n is the number of electrons (faradays mol^{-1}), A is the electrode area, and F = 96500 C $faraday^{-1}$. Equation 1 is valid, when only diffusion contributes to the flux at the electrode surface.

Cyclic Voltammetry

Cyclic voltammetry, just one type of voltammetric technique, is very suited to membrane studies and is very informative. Cyclic voltammetry involves cycling the potential of the working electrode in an unstirred solution, and measuring the resulting current. A triangular wave voltage is applied to the system. If the scan starts at a potential positive of

E°' for the reduction, only residual currents flow. As the electrode potential approaches E°', reduction begins and the current starts to flow. As the potential becomes more negative, the surface concentration of the species drops, leading to an increase in the flux to the surface, and hence an increase in the current. As the potential moves past E°', the surface concentration drops to near zero, the mass transfer (diffusion) to the electrode reaches a maximum rate, and then declines due to the depletion of the reducible species in the vicinity of the electrode. When the scan direction is reversed at the switching potential, the potential then sweeps in a positive direction. There is a large concentration of the oxidisable species in the vicinity of the electrode. As the potential becomes more positive, approaching and then passing E°', oxidation will occur resulting in an anodic peak. In this experiment, the characteristic peaked shape of the voltammogram occurs because the rate of variation of the potential was too rapid for the diffusion processes to maintain equilibrium with the bulk solution.

Each cyclic voltammogram is characterised by the following parameters: the separation of the cathodic and anodic peak potentials (E_{pa}-E_{pc}), the ratio of the peak currents (i_{pa}/i_{pc}), the cathodic half-peak potential ($E_{p/2}$), and the half-wave potential ($E_{1/2}$). The half-wave potential is related to the standard redox potential by

$$E_{1/2} = E^\circ + RT/nF \ln\left(\frac{D_{red}}{D_{ox}}\right)^{\frac{1}{2}} \qquad (2)$$

where D_{red} and D_{ox} are the diffusion coefficients for the reduced and oxidised species respectively, n is the number of electrons involved and the other terms have their usual meaning.

In a reversible system the rate of a charge transfer process is fast enough to maintain the laws of thermodynamics and the ratio of surface

concentrations of oxidant and reductant can be calculated from the Nernst equation. A reversible system has a voltammogram which satisfies the following criteria (where all symbols have their usual meaning):

i) E_p , the peak potential, is independent of v , the scan rate,

ii) $E_{pc} - E_{pa} = 2.2RT/n$ F mV at 25° C, and is independent of v ,

iii) $i_p / v^{1/2}$ is independent of v ,

iv) i_{pa} / i_{pc} is unity and is independent of v , and

v) $E_{1/2}$ is situated exactly midway between E_{pa} and E_{pc}.

When the charge transfer process is much slower than the diffusion rate, the electrode surface concentrations of the oxidised and reduced species, and therefore the current, is determined by kinetics alone. In this case the system does not obey the Nernst equation and is said to be irreversible. The cathodic peak is shifted more negatively and the anodic peak shifted more positively along the potential axis, with the peak separation so large that there is often no current observed on the return potential sweep. For a quasi-reversible system, the current is controlled by both diffusion and charge transfer kinetics. Note that if the product of a reversible charge transfer is destroyed by a rapid chemical reaction such that no reverse reaction can proceed, the response will be similar to that of an irreversible charge transfer reaction.

10.2.2 APPLICATION OF CYCLIC VOLTAMMETRY TO INVESTIGATIONS OF MODEL MEMBRANES

All measurements were made with a three-electrode potentiostatic circuit. The potentiostat (Amel, Model 554) was used to control the variable power supply. The signal generator consisted of a universal programmer (Princeton Applied Research, Model 175), which provided the triangular waves. The triangular potential sweep was usually applied at a scan rate of 200 mV s^{-1}. Current-voltage responses of the system were recorded on a X-Y

recorder. Negative potential and cathodic current coordinates in the resulting figures were directed to the left and downwards, respectively.

Throughout the investigation presented here, the potential was applied across the calomel electrode immersed in the trough, referred to as the back trough (ie. the one farthest from the microscope), and the reference electrode (RE), which was a calomel electrode placed in the front trough. The counter electrode (CE), a platinum wire, was placed in the front trough, close to the reference electrode. It should be noted that to minimise interference with the formation of membranes, the electrodes were all placed in the minor troughs. Generally, an oxidising agent or an equimolar solution of oxidising agent/reducing agent was added to the back trough. The species to be investigated was then added to the front trough. Tien, *et al.* [105] suggest that if the concentration of the species in the back trough is high enough, the modified BLM-aqueous solution interface in the front trough behaves like the conventional working electrode in the sense that the current is determined by the concentration gradient and diffusion in the front trough. Therefore, voltammograms represent the redox processes of the investigated species in the front trough, and the current is limited by its diffusion. It is important that there is sufficient modifier present so that the current is not limited. In some cases, the current/potential response of the system may be influenced by the concentration gradients in both troughs.

The potential of the *working electrode* (the BLM-aqueous solution interface in the front trough) in this setup is determined by consideration of the reaction at the calomel electrode in the back trough and also the reactions occurring at the BLM-aqueous solution interfaces in both troughs. All voltammograms from this work show the current measured at the working electrode plotted against the voltage applied between the working electrode and the reference electrode. The reactions occurring at the calomel electrodes on either side of the membrane cancel. Therefore, the

values on the potential scale are the difference between the potentials on either side of the membrane.

Usually a certain voltage exists across the membrane even if no voltage is applied from an external source, so that zero-current does not necessarily mean zero-voltage for the BLM. There may be a small potential difference between the calomel electrodes (which was shown to be less than 2 mV for the calomel electrodes used in these experiments) and redox equilibria in solutions on either side of the membrane may cause a potential difference. Therefore, in order to attain the zero-current point it may be necessary to apply an initial voltage to the membrane in order to compensate for the inherent voltage in the system.

To explain the observed membrane potential, the membrane is assumed to behave like an ideal electron conductor and the BLM as an electrical cell, where each membrane-aqueous solution interface is assumed to behave as a redox electrode. The observed current of a redox system is a measure of the rate of an electrochemical reaction and the Eyring equation [106] gives the relationship between the current and various parameters, such as the rate constant, transfer coefficient, applied voltage, redox potentials and concentrations of species. At equilibrium the current will be zero and substitution of this into the Eyring equation reduces it to the Nernst equation [106]:

$$\text{Applied voltage, } U = U_0 + \frac{RT}{nF} \ln \frac{[Ox]}{[Red]}$$

where U_0 is the standard redox potential of the redox couple and [Ox] and [Red] are the concentrations of the oxidised and reduced forms of the electroactive species. (F, R and T have their usual meanings.)

The electrical potential of each side of the membrane can be written in the same way, so that the overall potential generated by the transfer of electrons from a donor to an acceptor is

$$U = U_2 - U_1 = U_{0,2} - U_{0,1} + \frac{RT}{nF} \ln \frac{[Ox]_2[Red]_1}{[Red]_2[Ox]_1}$$

where U_1 and U_2 are the potentials of the two troughs, and $U_{0,1}$ and $U_{0,2}$ are the standard redox potentials for the two redox couples.

When [Ox] = [Red] in each trough the potential difference of the troughs, $\Delta U = U_m = U_{0,2} - U_{0,1}$, where U_m is the membrane potential measured under open circuit conditions ($i = 0$). When considering the cyclic voltammetry of BLMs at non-equilibrium, equations other than the Nernst equation must be considered [106].

The membrane potential, measured using a voltmeter (Keithley Instruments) or the potentiostat (with CELL OUT), agreed with the value of the applied potential at which the current was zero (read from the voltammogram). Therefore, when the potential difference between the two solutions on either side of the membrane was applied to the working electrode, no current flowed. When a negative potential is applied to the working electrode, it is driven to become more negative than the rest potential. Conversely, when a positive potential is applied, the working electrode becomes more positive than the rest potential.

10.2.3 MODIFICATION OF MEMBRANES

Current-voltage curves of unmodified membranes show that there is a linear relationship between membrane current and the voltage applied, so that Ohm's Law : $i = \frac{E}{R}$, is obeyed.

Results from studies investigating redox reactions in BLM systems suggest that on one side of the BLM electrons are donated to the membrane interface by electron donors, and on the other side of the membrane electrons are taken away from the interface by electron acceptors. A modifier acting as an electron donor or acceptor incorporated into the membrane phase transports the electrons across the bilayer. Examples of modifiers suitable for electron transfer investigations are discussed below.

TCNQ

In the early 1960s, 7,7,8,8-tetracyano-*p* -quinodimethane (TCNQ) was synthesised and found to be a new powerful electron acceptor, as it can gain an electron to become an anion radical [107]. TCNQ is a planar, symmetric molecule with π-delocalisation extending throughout the molecule [108]. It is generally accepted that it is the excitation of π electrons, which move throughout the whole molecule, that leads to the generation of mobile charge carriers. There is a possibility that molecular diffusion of TCNQ and/or $TCNQ^-$ is involved.

IODINE

The effect that iodine has on the the properties of membranes has been a subject for discussion since it was first noticed. Rosenberg and Jendrasiak[109,110] observed that the presence of iodine in the aqueous phase increased the conductivity of a BLM by several orders of magnitude. This was consistent with reports of Läuger, *et al.* [111], who suggested that the increase was due to formation of a molecular complex between the lipid and iodine, indicating that an electronic conduction mechanism involving redox reactions at the bilayer-aqueous solution interface could occur. It was thought that I_2 interacts with the polar groups of lecithin resulting in charge-transfer complexes in which the molecular ratio of components is

1:2 [112]. The two lecithin molecules in the complex act as donors and iodine acts as an acceptor. Studies of permeability and electrical properties led Finkelstein and Cass [113] to the conclusion that polyiodides (eg. I_3^- and I_5^-) are the major charge carriers in an ionic mechanism. Jain, *et al.* [114] made observations that were consistent with polyiodide-mediated electron transfer across the BLM. It is believed that the polyiodides penetrate the membrane and become electron sources. Liberman, *et al.* [115] supported the idea that an ionic mechanism was responsible for the increase in conductance. More recently, it was suggested by Bhowmik, *et al.* [116] that the total conductivity across the BLM is equal to the ionic conductivity outside the BLM plus the electronic conductivity inside the BLM.

OTHER ELECTRON CONDUCTORS

Tetrathiafulvalene (TTF), an electron donor [117], and ferrocene have also been found to act as electronic conductors in BLMs, but are not discussed in this work.

10.2.4 EXPERIMENTAL PROCEDURE

Planar bilayer membranes were formed as described in Chapter 7. When TCNQ was used as the modifier it was included in the BLM-forming solution. Iodine was usually present in the BLM-forming solution added to the troughs but to observe a non-ohmic voltammogram it was often necessary to add iodine (in an n-hexane solution) to the surface of the solution in the back trough after formation of the BLM.

After stirring a solution of TCNQ (solid) in a small amount of the membrane-forming solution the remaining solid was allowed to settle or centrifugation at low speeds was used to remove the undissolved solid material.

To generate the redox gradient, an equimolar solution of oxidising agent/reducing agent is added to the back trough and the redox couple to be investigated is added to the front trough. It was possible to have only an oxidising agent in the back trough when the species in the front trough was a reducing agent. The investigated species are usually of lower concentration than those present in the back trough.

A small magnetic stirrer was placed in an indent at the bottom of each trough and the solution in the trough was stirred vigorously for several minutes after the addition of the solutions containing the redox couples. The stirring was stopped and the voltammogram was recorded.

In electron transfer experiments, Tien has often added a saturated solution of KIO_4 to the back trough. As the solubility of potassium metaiodate (KIO_4) is 0.66 g /100 cm^3 of water (approx. 13° C) [118], the concentration of a saturated solution is 2.87×10^{-2} M.

CHAPTER 11 - RESULTS

Cyclic voltammograms given in this work show the current as measured between the calomel electrode in the back trough and the CE (platinum wire) in the front trough against the voltage applied between the calomel electrode in the back trough and RE (calomel electrode) in the front trough.

Membranes prepared from several different lipids will be assessed on the extent to which they act as a working electrode in a three-electrode arrangement. For discussion we consider first the lipid comprising the bilayer, then the modifier and finally the species under investigation, which is always placed in the front trough.

Voltammograms are compared with those obtained by Tien where possible, but it should be noted that Tien appears to make a correction for the membrane potential so that almost all his plots have zero current at zero voltage. Therefore the potential at which peaks occur is given relative to the potential at which i =0.

I. Natural lecithin

a) TCNQ-MODIFIED BLM

$Fe(CN)_6^{4-}$: The membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8). When the front trough contained 1.0×10^{-2} M $Fe(CN)_6^{4-}$ and the back trough contained 3.5×10^{-2} M $Fe(CN)_6^{3-}$ /$Fe(CN)_6^{4-}$ (Fig. 11.1) the plot was asymmetrical with almost no cathodic current. The anodic current reaches a plateau at 12μA cm^{-2}, which is usually indicative of a constant supply of oxidisable ions ($Fe(CN)_6^{4-}$) to the *working electrode.*

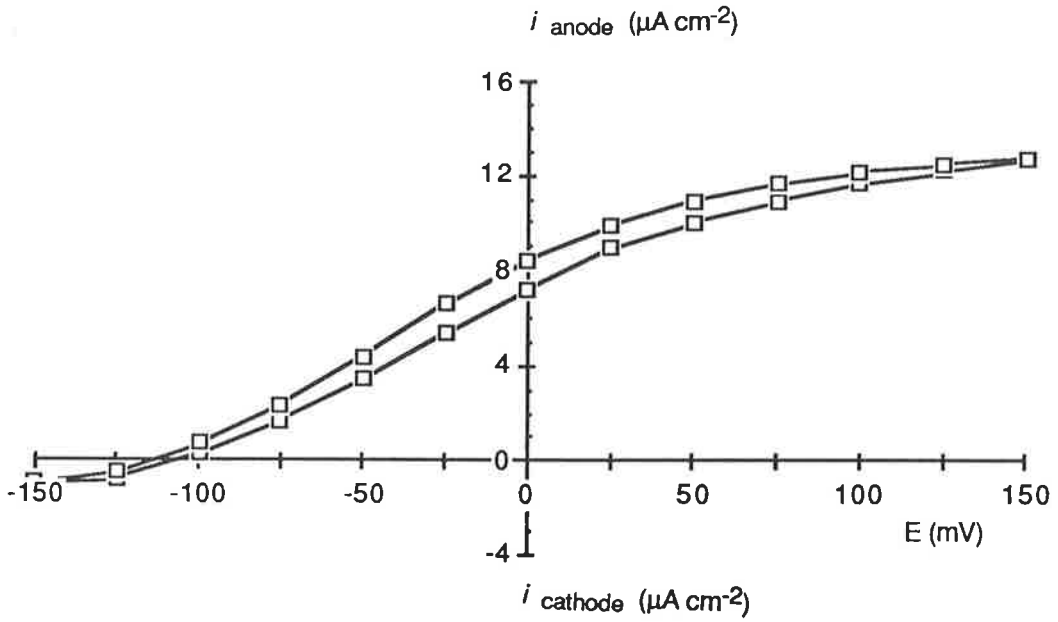


FIGURE 11.1 : Cyclic voltammogram of a TCNQ-modified natural lecithin BLM with a 3.5×10^{-2} M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ solution in the back trough and a 1×10^{-2} M $Fe(CN)_6^{4-}$ solution in the front trough. Diameter of aperture was 0.52 mm.
Electrolyte solution was 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}.

b) I_2-MODIFIED BLM

All the following systems were investigated using I_2-modified natural lecithin membranes. Unless otherwise stated the membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8).

i) **$Fe(CN)_6^{4-}$** : When I_2 was used as the modifier instead of TCNQ, almost all the voltammograms exhibited peaks, were symmetrical about (0,0), and the current was much higher (by at least an order of magnitude). In Figure 11.2 the back trough contained 3.5×10^{-2} M $Fe(CN)_6^{3-}$. The cyclic voltammogram of a system was sometimes asymmetrical, but after adding more I_2 to the back trough and forming a new membrane a more symmetrical plot resulted (Fig. 11.2(b)). From Figure 11.2, it appears that concentration of the species in the front trough is not the only factor determining the current, as a less concentrated solution

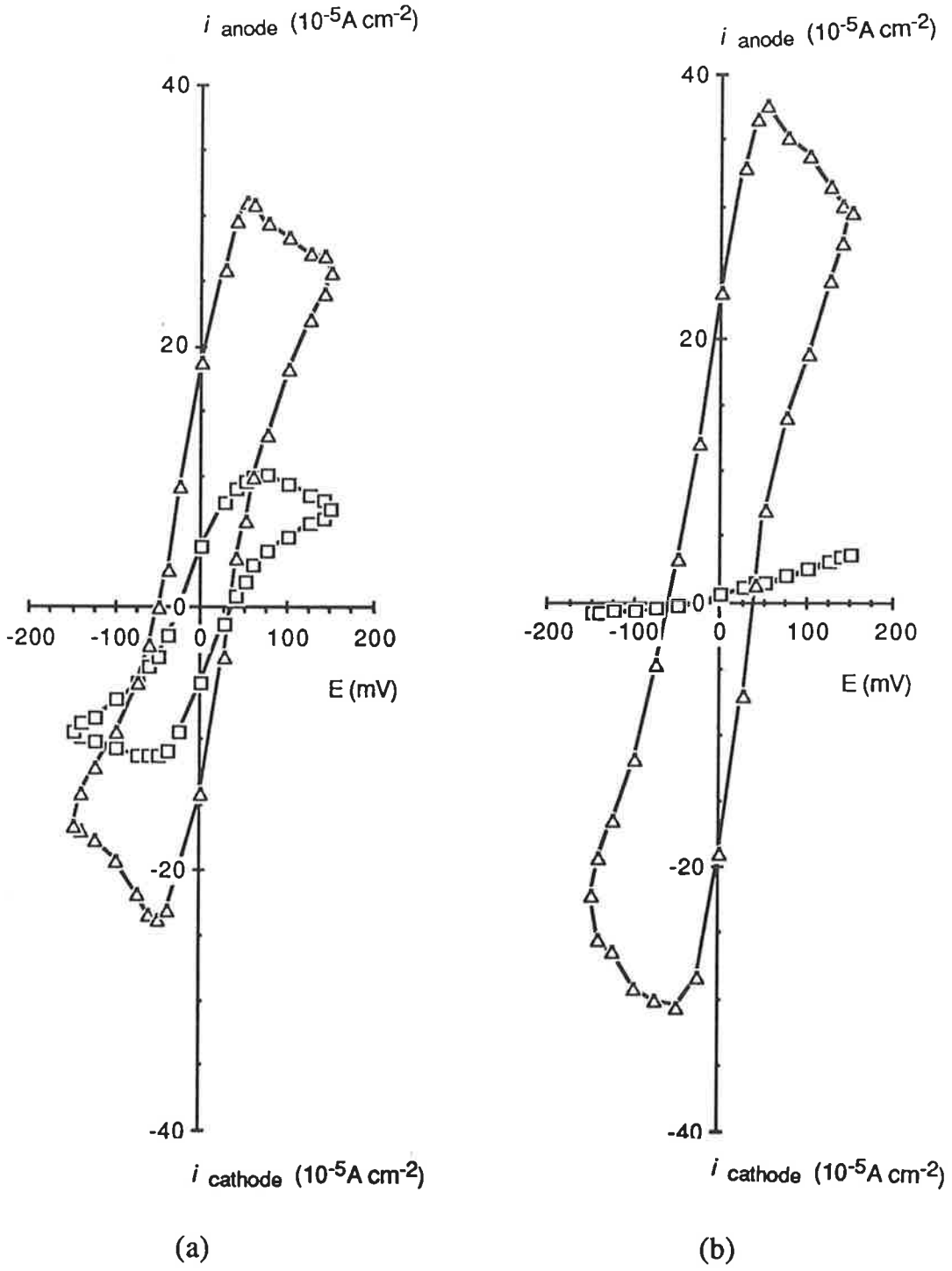


FIGURE 11.2 : Cyclic voltammograms of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) with a 3.5×10^{-2} M $Fe(CN)_6^{3-}$ solution in the back trough. (a) (□) 1×10^{-2} M $Fe(CN)_6^{4-}$ solution in the front trough ; (Δ) plot of second membrane formed with same system and solutions, but after the addition of I_2 in n-hexane to the surface of the solution in the back trough

(b) (□) 5×10^{-3} M $Fe(CN)_6^{4-}$ solution in the front trough ; (Δ) plot of second membrane formed with same system and solutions, but after the addition of I_2 in n-hexane to the surface of the solution in the back trough

Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameters of apertures were 0.52 mm.

may actually result in peaks of higher current (compare 11.2(a) and (b)). The indication is that the amount of I_2 in the BLM is a more significant factor. There were many cases when addition of more I_2 to the back trough resulted in a large increase in the current of subsequent membranes. In fact additions of more I_2 was often needed to even observe a non-ohmic plot.

When a very stable membrane was formed it was possible to change the scan rates and observe the change in the plot (Fig. 11.3). An essentially linear relationship is obtained when i_p is plotted against $\upsilon^{1/2}$ (Fig. 11.4) and as the voltammograms also show that i_{pa}/i_{pc} is approximately unity, it appears that the reaction is reversible [119]. It should be noted that the separation between the anodic and cathodic peaks is around 100 mV, and not 57 mV (that is, the peak separation is not equal to $2.2RT/n$ F mv, where n =1) as expected for a reversible system.

According to cyclic voltammetry theory, when the cathodic and anodic peak positions shift along the potential toward more negative and more positive potentials, respectively with higher scan rates, the rate limiting step of the process is no longer diffusion (neither in water phases in contact with BLM, nor the membrane itself), but charge transfer across the BLM [105]. As there is very little change in the peak positions for Figure 11.3, diffusion seems to be the rate-limiting step.

Symmetrical plots exhibiting peaks of comparable current are also obtained when KIO_4 (2.5×10^{-4} M) replaced $Fe(CN)_6^{3-}$ in the back trough (Fig. 11.5), under the same conditions.

These plots can be compared to I_2-modified natural lecithin/oxidised cholesterol membranes, with 0.1 M KCl and 0.01 M sodium acetate buffer (pH 5.5) as the bathing solution, which were formed by Krysinski and Tien [105]. With a calomel electrode inside the 10 cm^3

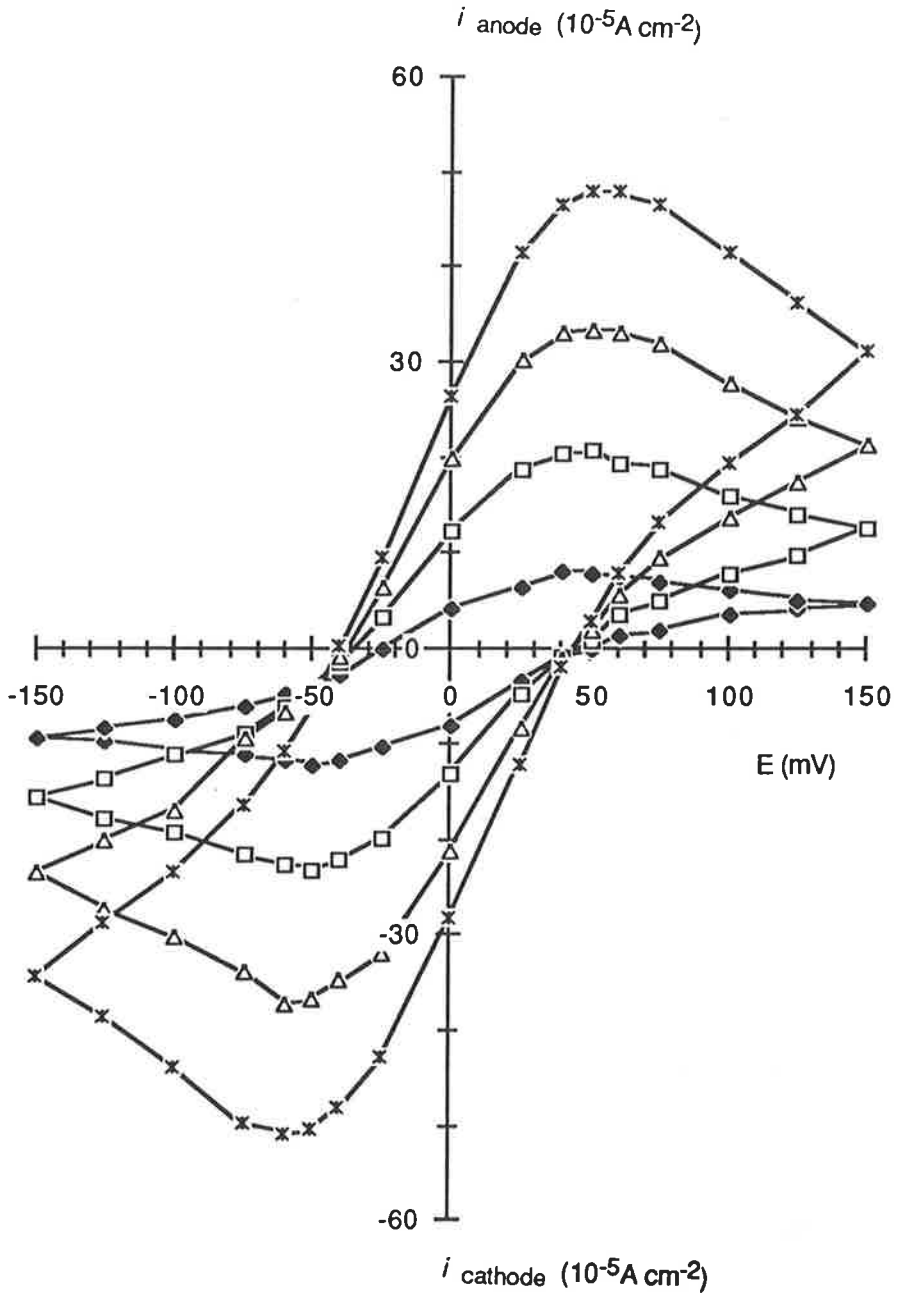


FIGURE 11.3 : Cyclic voltammograms of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) with a 3.5×10^{-2} M $Fe(CN)_6^{3-}$ solution in the back trough and a 5×10^{-3} M $Fe(CN)_6^{4-}$ solution in the front trough. (♦) Scan rate = 20 mV s^{-1} (□) Scan rate = 100 mV s^{-1} (Δ) Scan rate = 200 mV s^{-1} (*) Scan rate = 500 mV s^{-1} Electrolyte solution was 0.1 M CH_3COONa/0.1 M KCl. Diameter of aperture was 0.52 mm.

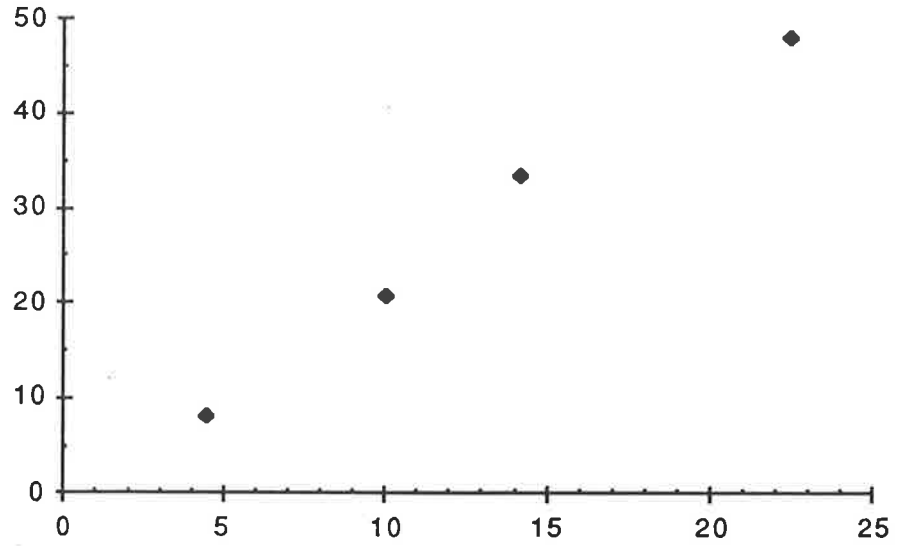


FIGURE 11.4 : Plot of peak current (i_p) against (scan rate)$^{1/2}$ when the scan rate is varied from 20 mV sec^{-1} to 500 mV sec^{-1}.

Teflon cup, which contained 100 mm^3 of saturated KIO_4 solution, and reference and counter electrodes placed outside the cup in 5×10^{-4} M $K_4Fe(CN)_6$, a peaked voltammogram was obtained. With a scan rate of 100 mV s^{-1}, i_{anod} peak (120-130 mV) occurred at 44×10^{-5}Acm^{-2} and i_{cath} peak (-90-100 mV) at -27×10^{-5}Acm^{-2}. The peak separation is higher than that found for the voltammograms presented here.

ii) **$(NH_4)_2SO_4.FeSO_4.6H_2O$** : With KIO_4 (100 mm^3 of saturated solution ; approximately 1.2×10^{-4} M) in the back trough and 5×10^{-4} M $(NH_4)SO_4.FeSO_4.6H_2O$ in the front trough, a peaked symmetrical voltammogram resulted (Fig. 11.6).

iii) **KI** : Plots exhibiting non-ohmic behaviour were obtained with the modified membrane in buffer only, but the current was very low. Addition of KIO_4 to the back trough resulted in the current-voltage plot

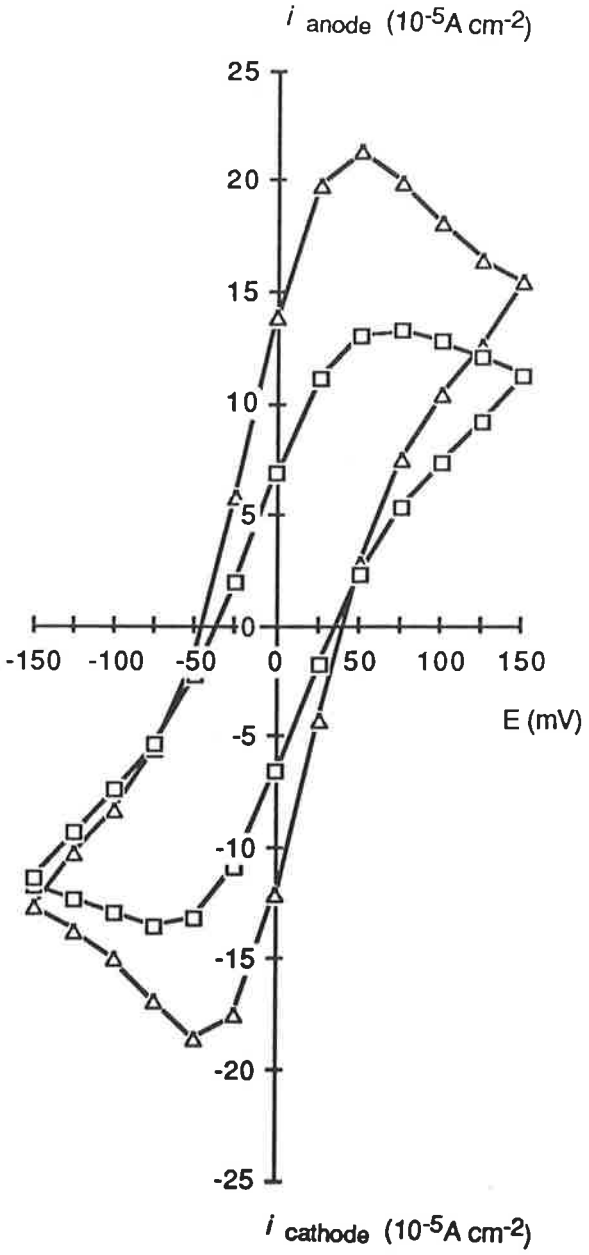


FIGURE 11.5 : Cyclic voltammograms of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) with a 2.5×10^{-4} M KIO_4 solution in the back trough and 5×10^{-3} M $Fe(CN)_6^{4-}$ solution in the front trough. Conditions were exactly the same for both plots; they were obtained on consecutive days using the same setup (ie. same Teflon septum and therefore same aperture), but the solutions were replaced after the setup was rinsed with chloroform.
Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameter of aperture was 0.52 mm.

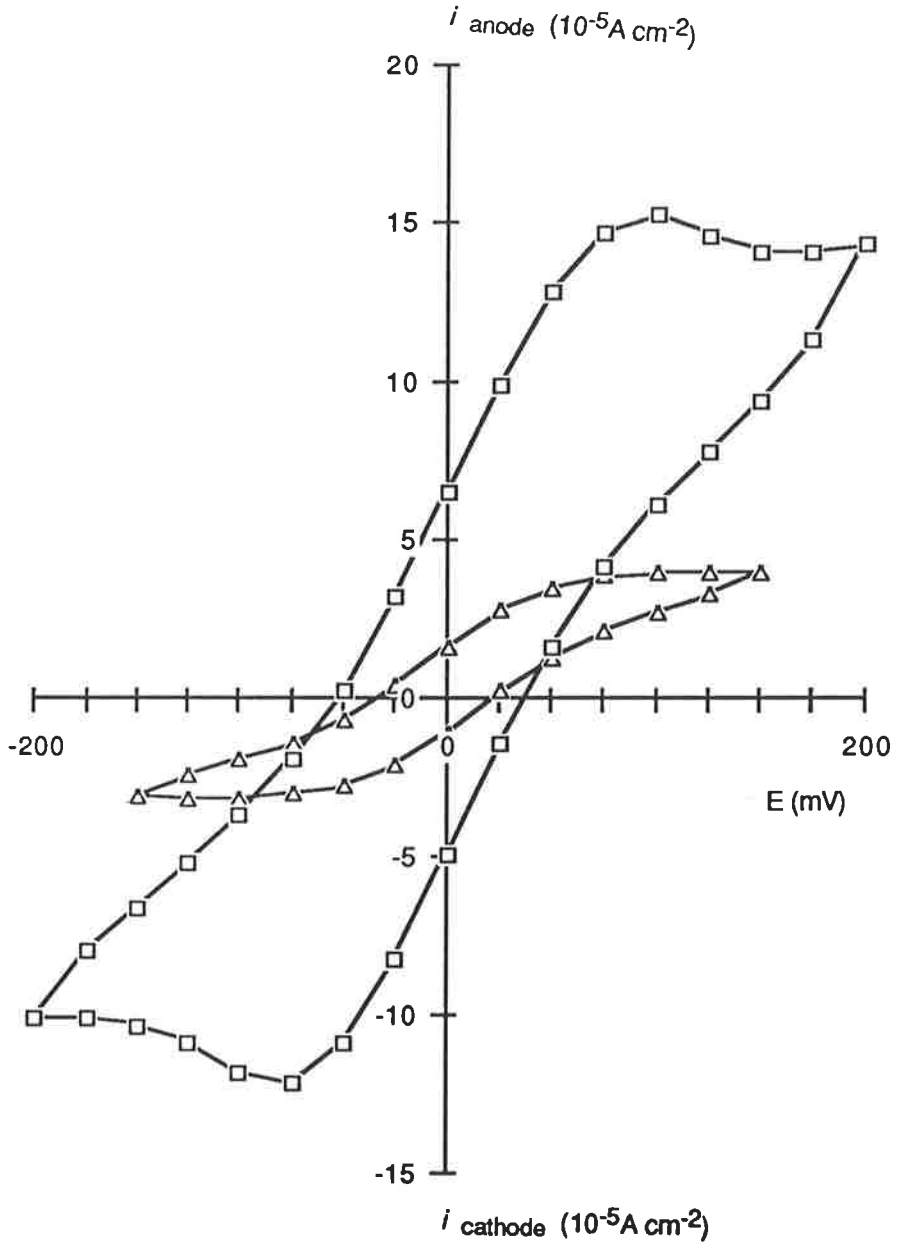


FIGURE 11.6 : Cyclic voltammograms of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) : (Δ) no redox couples in troughs (□) with a 1.2×10^{-4} M KIO_4 solution in the back trough and a 5×10^{-4} M $(NH_4)_2SO_4.FeSO_4.6H_2O$ solution in the front trough.
Electrolyte solution was 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}.
Diameter of aperture was 0.52 mm.

becoming more ohmic, while the current remained at a low level. Addition of KI to the front trough (concentration 5×10^{-4} M) greatly increased the current and either resulted in the plot becoming completely ohmic (Fig. 11.7(a)) or resulted in peaks in both the anodic and cathodic current (Fig. 11.7(b)). With a scan rate of 200 mV s^{-1}, i_{anod} peak (80 to 90 mV) occurred at $24\times10^{-5}Acm^{-2}$ and i_{cath} peak (-70 to -80 mV) at $-15\times10^{-5}Acm^{-2}$.

Peaked voltammograms were more likely if more I_2 was added to the surface of the solution in the back trough, either before or after formation of a new membrane (Fig. 11.8 and 11.9), and the magnitude of the current increased as expected.

These plots can be compared to I_2-modified natural lecithin/oxidised cholesterol membranes, with 0.1 M KCl/0.01 M sodium acetate buffer (pH 5.5) as the bathing solution, which were formed by Krysinski and Tien [105]. With a calomel electrode inside the 10 cm^3 Teflon cup, which contained 100 mm^3 of saturated KIO_4 solution, and reference and counter electrodes placed outside the cup in 5×10^{-4} M KI an asymmetrical peaked voltammogram was obtained. With a scan rate of 100 mV s^{-1}, i_{anod} peak (90-100 mV) occurred at $22\times10^{-5}Acm^{-2}$ and i_{cath} peak (-60-70 mV) at $-14\times10^{-5}Acm^{-2}$.

iv) **KIO_4 / KIO_3** : With a 10:1 molar ratio of KIO_4/KIO_3 solution in the back trough and a 1:10 molar ratio of KIO_3/KIO_4 in the front trough, voltammograms of the I_2-modified membranes exhibit non-ohmic behaviour, after more I_2 is added to the surface of the solution in the back trough (Fig. 11.10(a)).

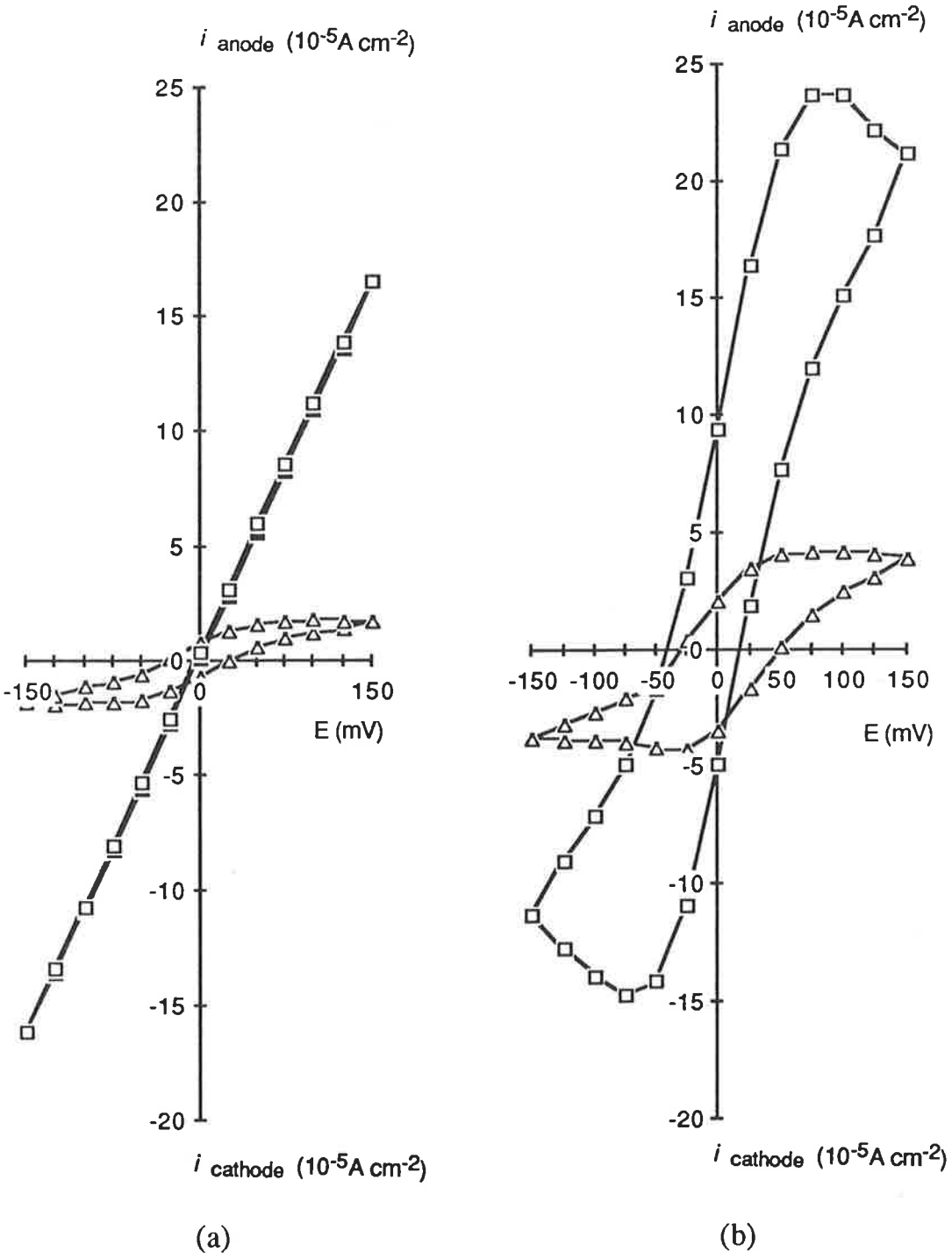


FIGURE 11.7 : Cyclic voltammograms of an I_2-modified natural lecithin BLM : (Δ) no redox couples present in the troughs (□) 1.15×10^{-4} M KIO_4 solution in the back trough and 5×10^{-4} M KI solution in the front trough. (a) and (b) were obtained on different days with different setups (ie. different apertures).
Electrolyte solution was 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}.
Diameters of apertures were 0.52 mm.

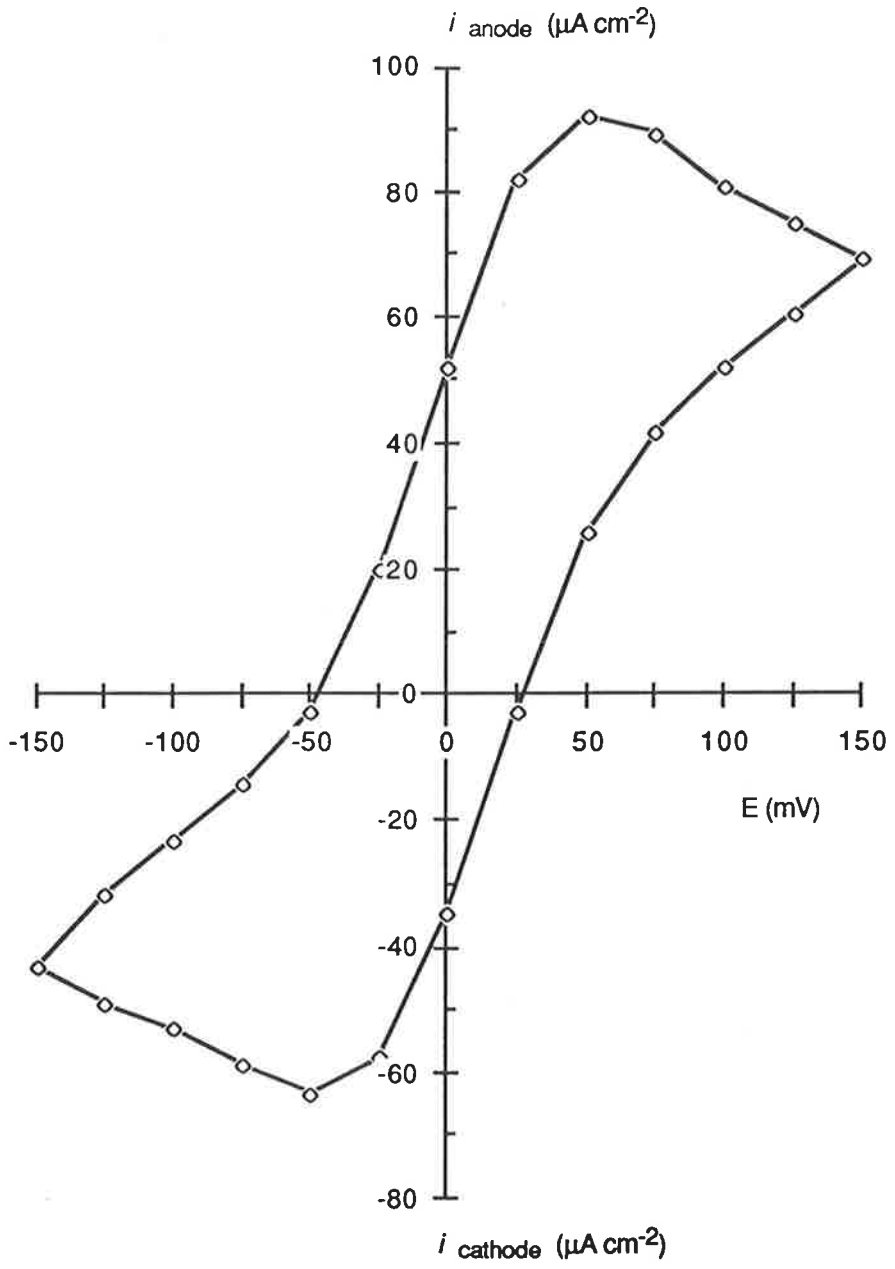


FIGURE 11.8 : Cyclic voltammogram of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) with a 1.6×10^{-4} M KIO_4 solution in the back trough and a 5.5×10^{-4} M KI solution in the front trough. It was necessary to add more I_2 to the back trough after formation of the BLM in order to obtain a peaked voltammogram of significant current.
Electrolyte solution was 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameter of aperture was 0.47 mm.

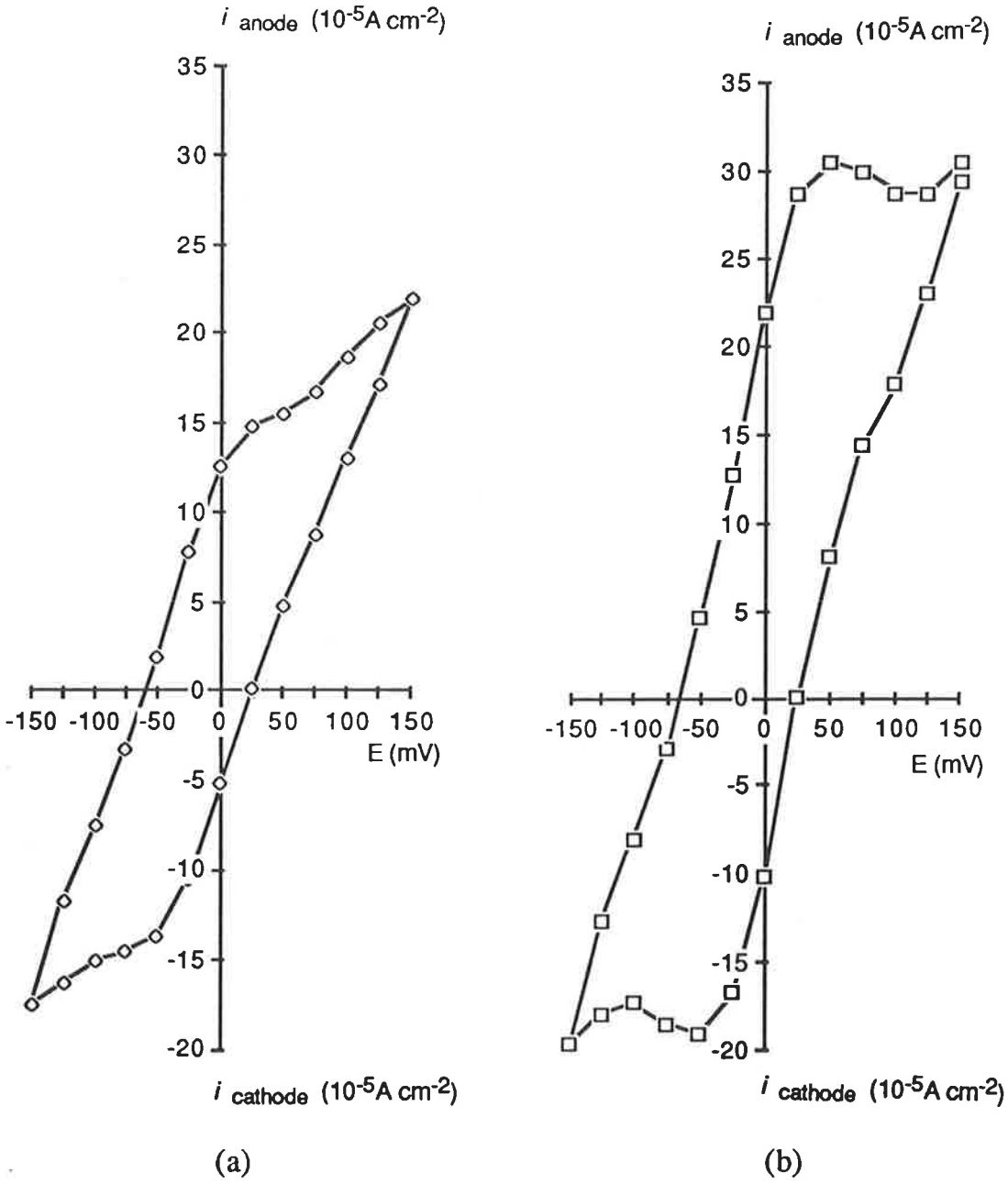


FIGURE 11.9 : Cyclic voltammograms of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) with 2.5×10^{-4} M KIO_4 solution in the back trough and 5.5×10^{-4} M KI solution in the front trough. The plots were obtained on different days with different setups with (a) diameter of the aperture used was 0.52 mm (b) diameter of the aperture used was 0.47 mm.
Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}.

v) **Dehydroascorbic acid / Ascorbic acid** : With KIO_4 (2.5×10^{-4} M) in the back trough and 5.5×10^{-4} M ascorbic acid in the front trough, the voltammogram of the system shows slight deviation from ohmic behaviour (Fig. 11.10(b)). It was probably necessary to add more I_2 to the back trough, as the current was very low. Peaked voltammograms of considerable current were obtained by Krysinski and Tien [105] under similar conditions.

vi) **No redox couples present** : Note that it was often observed that even when no redox couples are added to the buffer solution, the I_2-modified natural lecithin membranes exhibit non-ohmic behaviour. For I_2-modified membranes, the voltammograms of the system usually have peaks, but once an electroactive species or redox couple is added to the back trough the voltammogram may exhibit more ohmic behaviour, where the current is directly proportional to the voltage applied (Fig. 11.11). While this was a consistent and interesting phenomenon, the currents are very low and further studies are necessary before this could be considered to be significant.

II. Natural lecithin / oxidised cholesterol

a) TCNQ-MODIFIED BLM

Q/H_2Q : Numerous quininoid compounds are known to play important roles in biomembranes and quinhydrone is the simplest of those that have been characterised electrochemically. Quinhydrone consists of quinone and hydroquinone at equal concentrations.

Unless otherwise stated the membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8). For systems where a TCNQ-modified

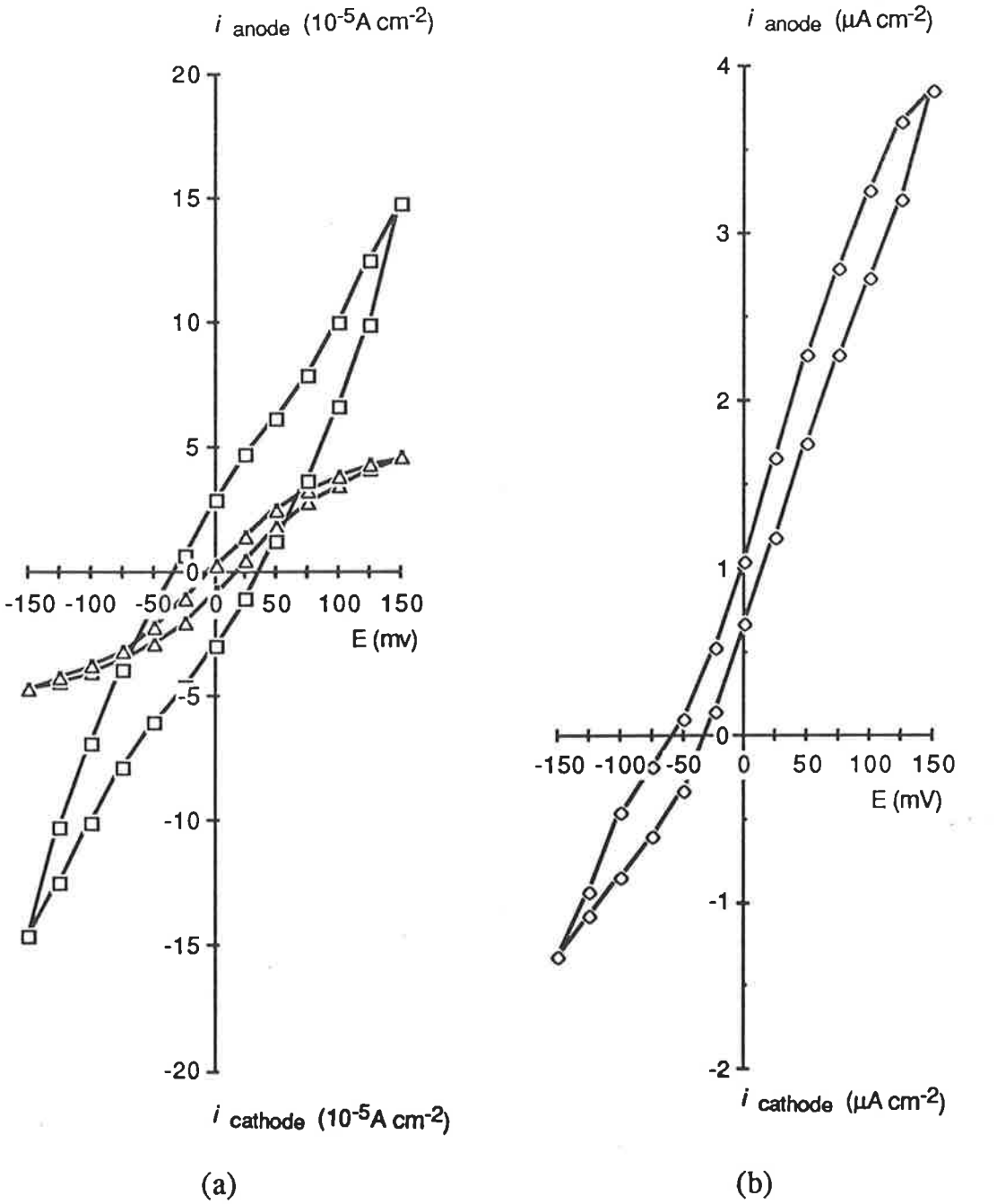


FIGURE 11.10 : (a) Cyclic voltammogram of an I_2-modified natural lecithin BLM (I_2 in only lipid solution added to back trough) with a 3.4×10^{-3} M KIO_4/3.1×10^{-4} M KIO_3 solution in the back trough and a 3.4×10^{-4} M KIO_4/3.1×10^{-3} M KIO_3 solution in the front trough. Two plots obtained with the same setup and without changing the solutions, but from different membranes with the addition of more I_2/n-hexane to the surface of the back trough resulting in higher current. (b) Cyclic voltammogram of an I_2-modified natural lecithin BLM with a 2.5×10^{-4} M KIO_4 solution in the back trough and a 5.5×10^{-4} M ascorbic acid solution in the front trough.
Electrolyte solutions for both setups were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameters of apertures were 0.52 mm.

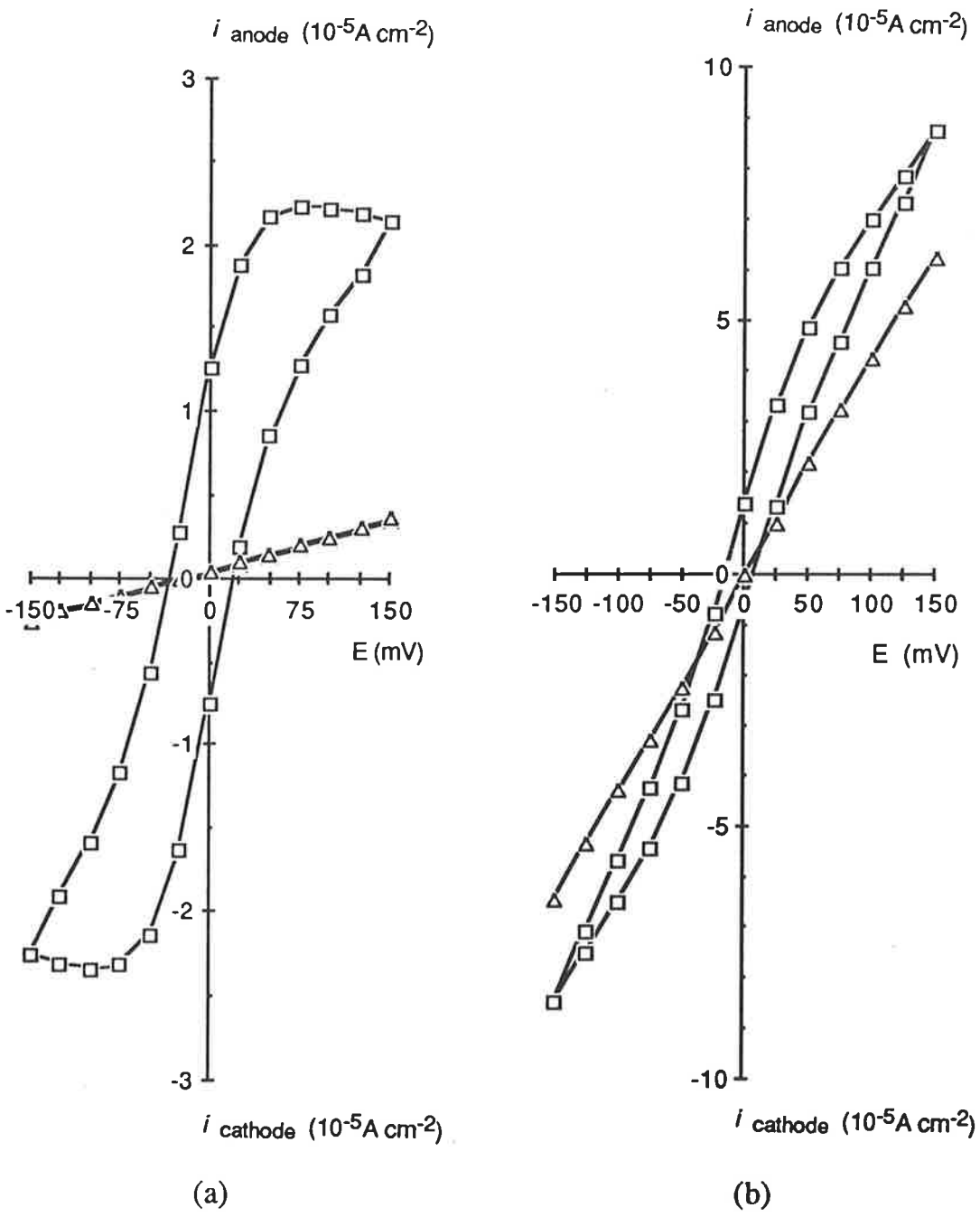


FIGURE 11.11 : Cyclic voltammograms of an I_2-modified natural lecithin BLM : (a) (□) no redox couples present in the troughs (Δ) with a $Fe(CN)_6^{3-}$ solution in the back trough ; (b) (□) no redox couples present in the troughs (Δ) with a 2.5×10^{-4} M KIO_4 solution in the back trough.
Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}.
Diameters of apertures were 0.52 mm.

membrane separates an equimolar solution of $Fe(CN)_6^{4-}/Fe(CN)_6^{3-}$ (3.5×10^{-2} M) in the back trough and quinhydrone (2.5×10^{-3} M) in the front trough, zero current flows at a value around -120 mV (Fig. 11.12). The current is quite low; the highest I_{anod} obtained was about 2.4 μAcm^{-2}. The plots are asymmetrical with the anodic current being much higher, that is, oxidation takes place more readily than reduction. It appears that larger negative potentials should have been applied in order to observe a cathodic peak.

The low current suggests that there is very little TCNQ in the BLMs to transport the electrons across the bilayer.

As the back trough contained an equimolar concentration of ferri-/ferrocyanide and the front trough contains quinhydrone, which consists of equal amounts of quinone and hydroquinone, it is assumed that the solutions in each trough attain a potential close to E° for that particular system.

For the quinhydrone system, $Q + 2H^+ + 2e^- \rightarrow H_2Q$:

$$E_Q = E_Q^\circ + (RT/F) \ln a_H$$

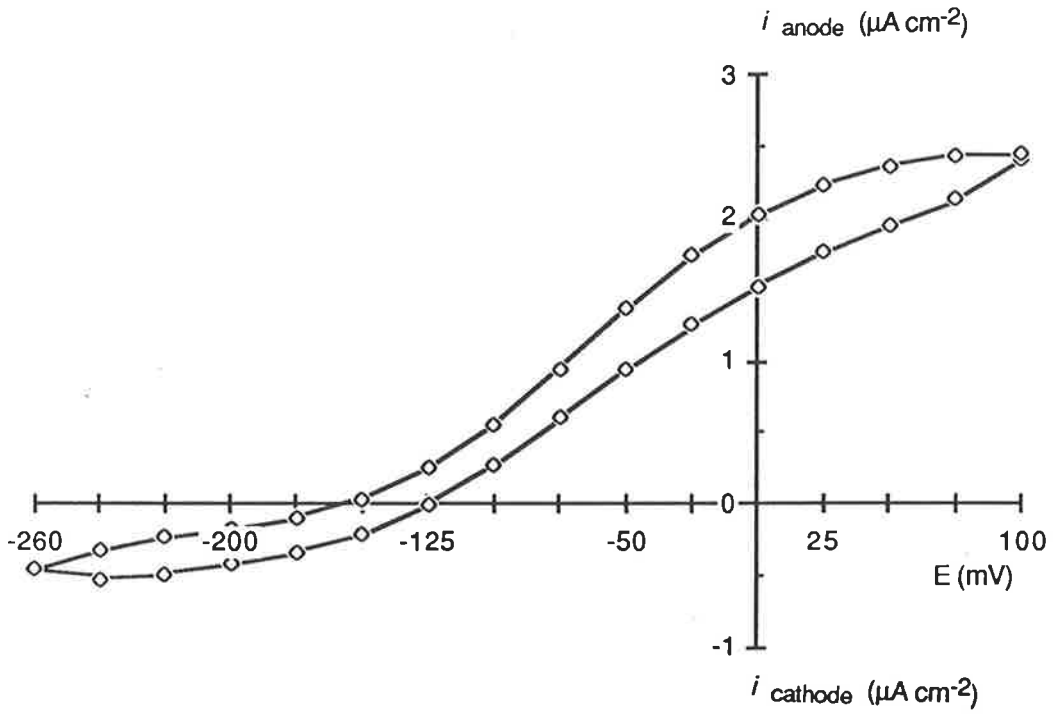
$$= E_Q^\circ + (RT/F) \ln [H^+]$$

$$= 0.69976 \text{ volts} + [(8.314 \text{ JK}^{-1}\text{mol}^{-1} \times 298^\circ \text{ K})/96500 \text{ C}] \ln 10^{-pH} \quad \text{at } 25^\circ \text{ C}$$

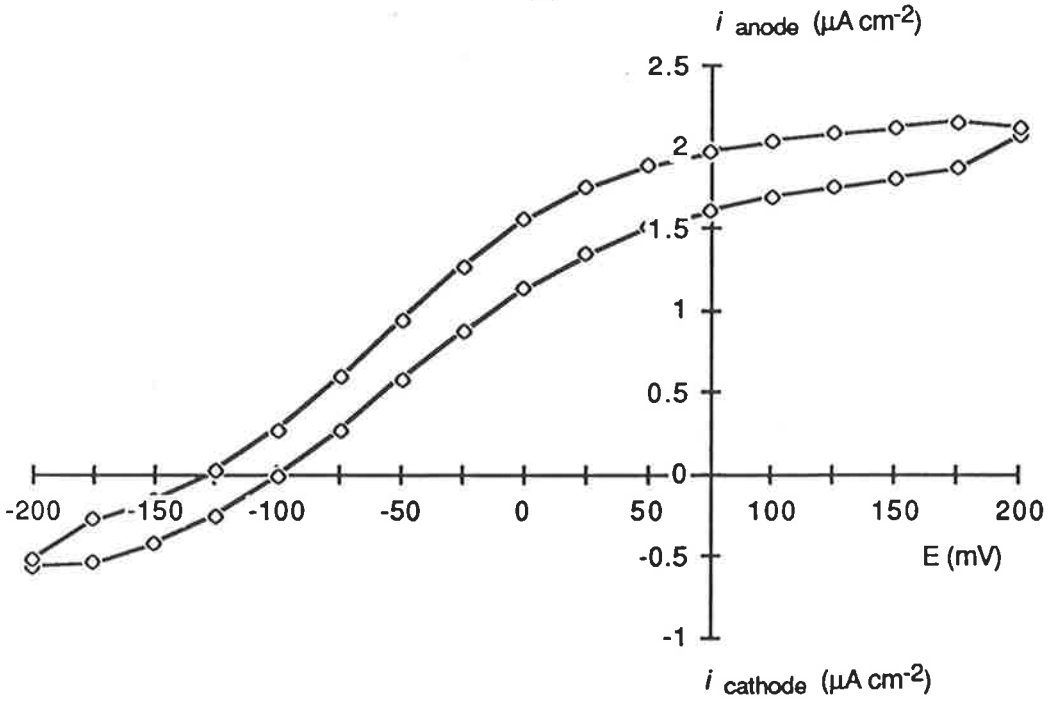
$$= 0.4751 \text{ volts , at pH 3.8.}$$

$$= 0.2268 \text{ volts , at pH 8.}$$

E° of equimolar $[Fe(CN)_6^{3-}]/[Fe(CN)_6^{4-}]$ is 0.36 volts, when measured against a hydrogen electrode.



(a)



(b)

FIGURE 11.12 : (a) and (b) : Cyclic voltammograms of a TCNQ-modified natural lecithin/oxidised cholesterol BLM with a 3.5×10^{-2} M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ solution in the back trough and a 2.5×10^{-3} M BQ/H_2Q solution in the front trough. Plots were obtained over two days with different setups (ie. different Teflon septums and therefore different apertures).
Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameters of apertures were 0.52 mm.

The membrane potential is the potential difference between the two redox couples ($E^{\circ}_{quinone/hydroquinone} - E^{\circ}_{ferri\text{-}ferrocyanide}$ = (0.23 - 0.36) volts = -0.13 volts, at pH 8).

This voltammogram can be compared with a TCNQ-modified natural lecithin/oxidised cholesterol membranes, with 0.1 M KCl as the bathing solution, which were formed by Tien [120]. With a calomel electrode inside the 10 cm^3 Teflon cup, which contained an equimolar $Fe(CN)_6^{3-}$ /$Fe(CN)_6^{4-}$ solution, and reference and counter electrodes placed outside the cup in 5×10^{-4} M quinhydrone a peaked voltammogram was obtained. With a scan rate of 200 mV s^{-1}, i_{anod} peak (80 to 90 mV) occurred at 0.17×10^{-5} Acm^{-2} and i_{cath} peak (-80 to-90 mV) at $-0.17\times10^{-5}Acm^{-2}$.

Another comparison can be made between this system and I_2-modified natural lecithin/oxidised cholesterol membranes, with 0.1 M KCl and 0.01 M sodium acetate buffer (pH 5.5) as the bathing solution, which were formed by Krysinski and Tien [105]. With a calomel electrode inside the 10 cm^3 Teflon cup, which contained 100 mm^3 of saturated KIO_4 solution, and reference and counter electrodes placed outside the cup in 4.63×10^{-3} M hydroquinone a peaked voltammogram was obtained. With a scan rate of 100 mV s^{-1}, i_{anod} peak (80 to 90 mV) occurred at $18\times10^{-5}Acm^{-2}$ and i_{cath} peak (-80 to-90 mV) at $-20\times10^{-5}Acm^{-2}$.

b) I_2-MODIFIED BLM

$Fe(CN)_6^{4-}$: The membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8). The presence of cholesterol in the BLM-forming solution did not significantly change the peaked voltammograms from those obtained when there was no oxidised cholesterol in the BLM-forming solution. The plot in Figure 11.13 is an example of the behaviour

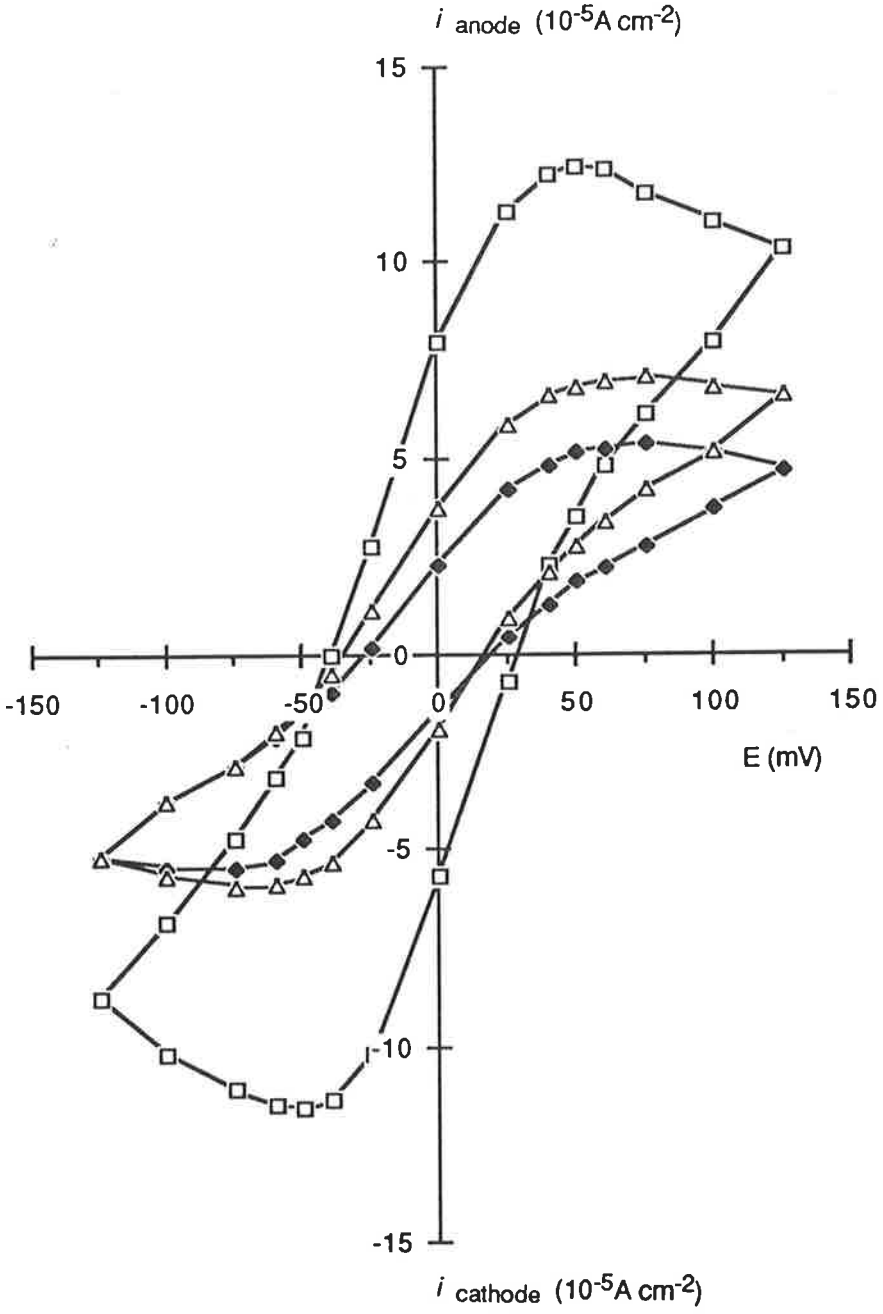


FIGURE 11.13 : Cyclic voltammograms of an I_2-modified natural lecithin/oxidised cholesterol BLM (I_2 in only lipid solution added to back trough) with a 2.5×10^{-4} M KIO_4 solution in the back trough and a 5×10^{-3} M $Fe(CN)_6^{4-}$ solution in the front trough. (Δ) initial plot with *i* increasing with continued scanning (□) after adding more I_2 to the surface of the back trough (♦) with continuous scanning *i* decreased to this plot.
Electrolyte solution was 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameter of aperture was 0.52 mm.

of I_2-modified membranes, where as more I_2 in n-hexane was added to the surface of the solution in the back trough, the current increased, stabilised and then decreased to a lower value with continuous scanning.

Monoolein

TCNQ-MODIFIED BLM

All the following systems were investigated using TCNQ-modified monoolein membranes. Unless otherwise stated the membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8).

Dehydroascorbic acid / Ascorbic acid : TCNQ-modified BLM were formed in 0.0998 M NaCl. The back trough contained an equimolar solution of ferro-ferricyanide at the same concentration (5×10^{-3} M) as the solution of ascorbic acid on the other side of the BLM. The resulting voltammogram indicates that there is no net current at -100 mV (Fig. 11.14). A steady state appears to be reached in the anodic current; this is usually indicative of a stirred solution where the supply of reacting species is continuous. The anodic current becomes constant at +34.6 μAcm^{-2}, while the cathodic current reaches -23.1 μAcm^{-2} in the range scanned. It should be noted that the current of this voltammogram is considerably higher than that obtained with most other BLMs modified with TCNQ.

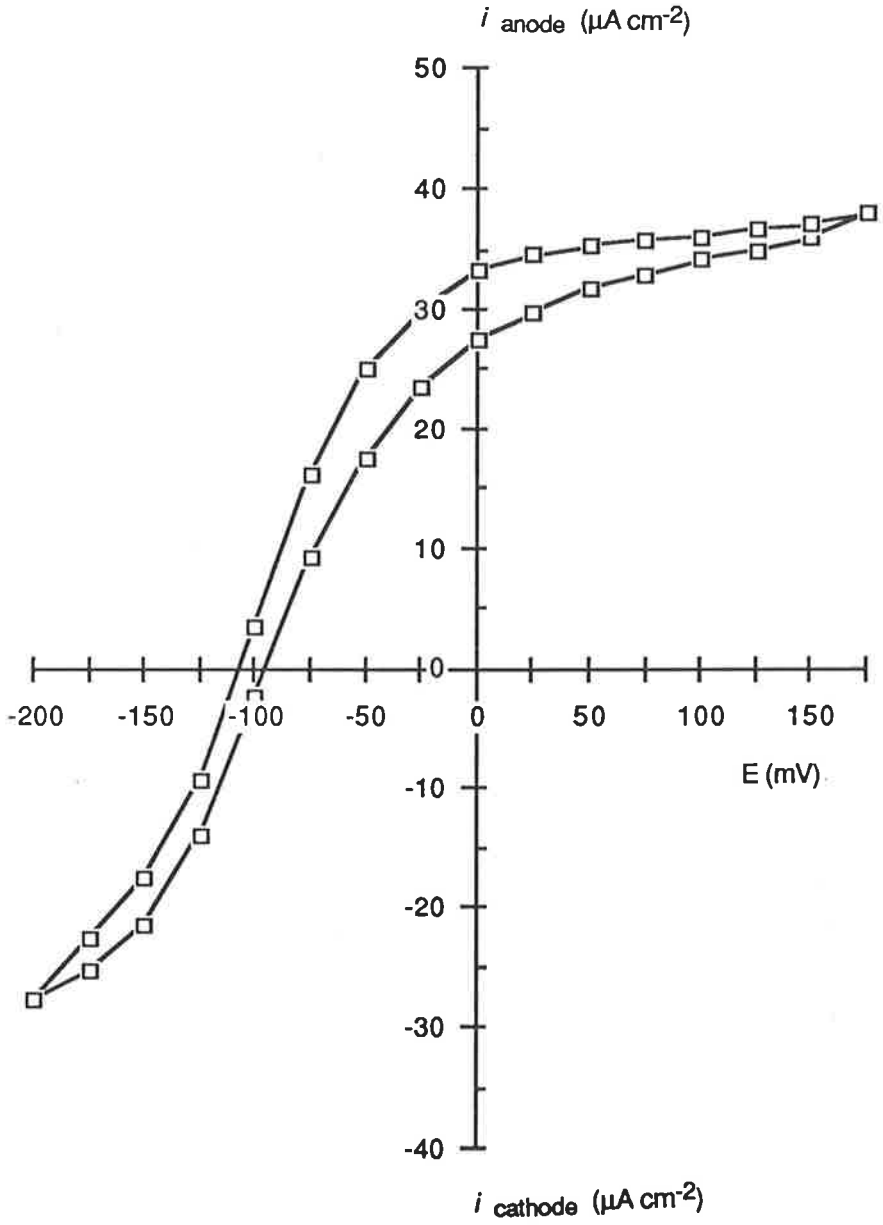


FIGURE 11.14 : Cyclic voltammogram of a TCNQ-modified monoolein BLM with a 5×10^{-3} M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ solution in the back trough and a 5×10^{-3} M ascorbic acid solution in the front trough.
Electrolyte solution was 0.0998 M NaCl. Scan rate = 200 mV s^{-1}. Diameter of aperture was 0.47 mm.

PE / PS

a) TCNQ AS THE MODIFIER

Dehydroascorbic acid / Ascorbic acid : The membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8). TCNQ-modified PE/PS (2:1) membranes were formed with $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ (3.5×10^{-2} M) in the back trough. As the concentration of ascorbic acid in the front trough increased from 5.0×10^{-3} M to 1.5×10^{-2} M the rest potential (where no current flows) read from the voltammograms varied from -100 mV to -175 mV. Voltammograms obtained with this system (buffer solution at pH 8) are asymmetrical with an anodic current (indicating that oxidation occurred) but very little, if any, reduction (Fig. 11.15). The currents are quite low.

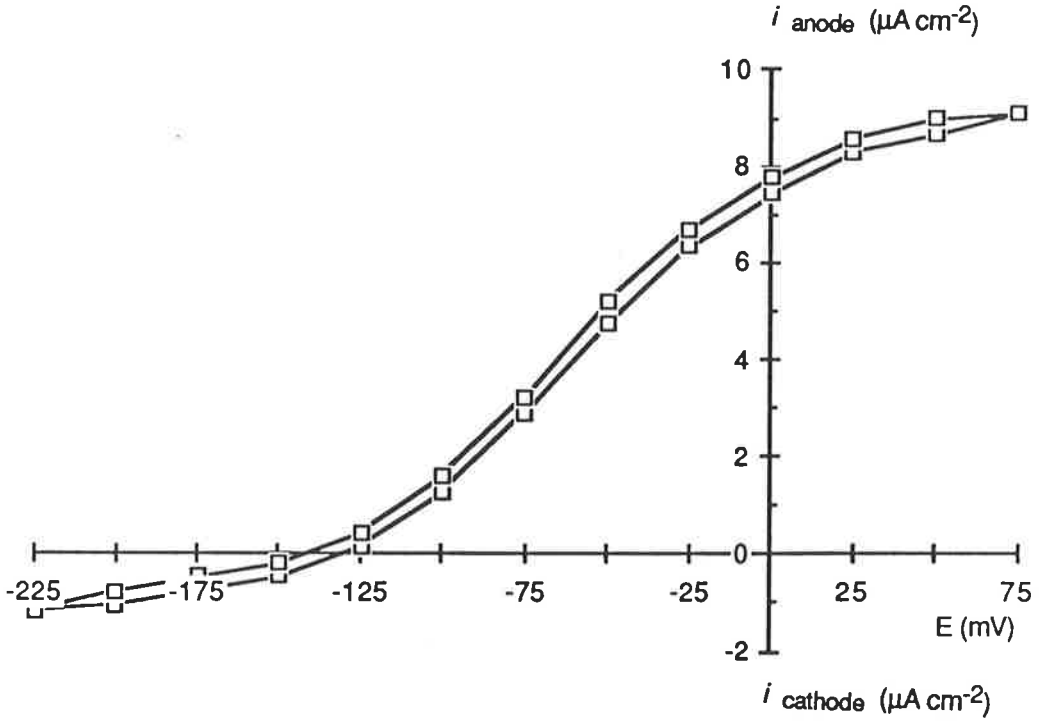


FIGURE 11.15 : Cyclic voltammograms of a TCNQ-modified PE/PS (2:1) BLM with a 3.5×10^{-2} M $Fe(CN)_6^{3-}/Fe(CN)_6^{4-}$ solution in the back trough with 1×10^{-2} M ascorbic acid solution in the front trough.
Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}.
Diameters of apertures were 0.47 mm.

b) I_2-MODIFIED BLM

All the following systems were investigated using I_2-modified PE/PS membranes. Unless otherwise stated

KIO_4 / KIO_3 : The membranes were formed in 0.1 M CH_3COONa/0.1 M KCl (pH 8). With a 10:1 molar ratio of KIO_4/KIO_3 solution in the back trough and a 1:10 molar ratio of KIO_4/KIO_3 in the front trough, voltammograms of the I_2-modified membranes exhibit no peaks in the range scanned but the currents are significant (Fig. 11.16).

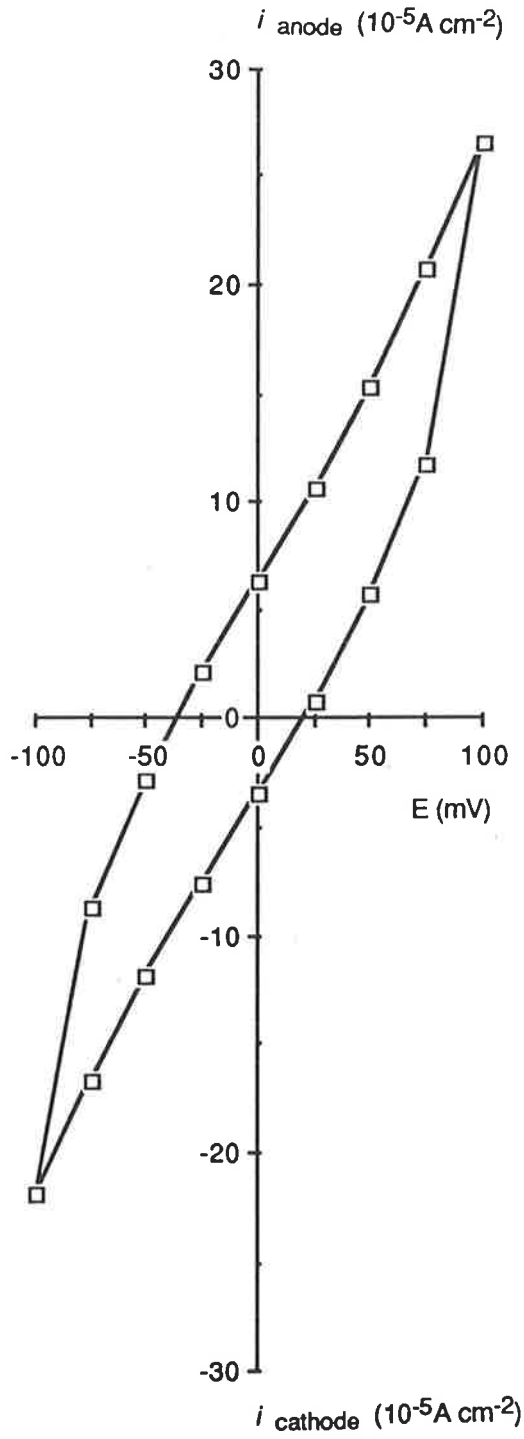


FIGURE 11.16 : Cyclic voltammogram of an I_2-modified PE/PS (2:1) BLM (I_2 in only lipid solution added to back trough) with a 3×10^{-3} M KIO_4/4.3×10^{-4} M KIO_3 solution in the back trough and a 3×10^{-4} M KIO_4/4.3×10^{-3} M KIO_3 solution in the front trough. Electrolyte solutions were 0.1 M CH_3COONa/0.1 M KCl. Scan rate = 200 mV s^{-1}. Diameter of aperture was 0.47 mm.

CHAPTER 12 - DISCUSSION

In electron transfer experiments the BLM usually separates an acceptor-rich side and a donor-rich side, which are referred to as the acceptor and donor sides by many researchers [121]. This was the case for some systems presented here, but in general the back trough contained a reasonably high concentration of redox couples (both acceptor and donor), while a lower concentration of another redox couple (or only the donor species) was present in the front trough. As mentioned earlier, it is assumed that when electron transfer occurs it is the BLM-aqueous solution interface in the front trough that functions as the *working electrode*. A higher concentration of redox species in the back trough should ensure that the current is not limited by processes occurring there. Therefore, current and the extent of deviation from the ohmic plot is dependent on the amount of modifier present in the BLM and the concentration of the redox species in the front trough [105,106].

During the cathodic scan (applied potential is increasingly negative), more and more electrons are available for reduction to the species adjacent to the BLM. This means that the BLM becomes a stronger reducing electrode. Conversely, as the potential is increased in the positive direction, the BLM becomes a better oxidising electrode, with positive holes increasingly ready for oxidation [119].

It is difficult to compare voltammograms of systems prepared on a different days, as the concentration of the solutions may be similar but the amount of modifier present greatly affects the current-voltage behaviour. To further complicate comparisons, it is unlikely that the concentration of the modifier in the BLM-forming solution is the same as that of the modifier actually incorporated into the BLM [120].

"The midpoint potential always corresponded to the Nernst potential of the BLM partitioned cell"[122,123]. Tien observed [106] that the extrapolated value for the membrane potential with equal molar concentrations of ascorbic acid and ferri/ferrocyanide on opposite sides of the BLM agrees with the difference of the two standard potentials (that is, about 300 mV). This ignores the fact that the standard potential to which he refers is measured with equimolar solutions of the reductant and the oxidant in the same trough. In experiments where ascorbic acid is added to the trough the concentration of dehydroascorbic acid is very low and certainly not equal to the concentration of ascorbic acid. If the modified membrane behaves as a ideal electron conductor, the measured potential difference should be equal to the difference of the equimolar redox couples on opposite sides of the barrier.

With few exceptions voltammograms reported by Tien and co-workers have zero current when zero voltage is measured between the calomel electrode in the back trough and the reference electrode (in the front trough). While this has been the case for many iodine-modified membrane systems, it certainly is not true for many other systems. Tien has not mentioned that the zero on the potential axis in his diagrams refers to the rest potential, leading to the observation that for the majority of his experiments, zero current does indeed occur when there is no applied voltage.

During the investigation of many of these systems small residual currents were obtained and too much emphasis should not be placed on the interpretation of these plots.

Throughout the investigation, TCNQ-modified membranes gave plots showing less current than the iodine-modified membranes. The majority of the plots were above the level of residual current, but were

dependent on the amount of electron carrier present in the membrane. Although the currents obtained by Tien and co-workers are comparable for most systems there is a possibility that the additional solvent present in black lipid films assists in attaining a higher concentration of TCNQ in the BLM.

When the BLM-forming solution contained a fine suspension of very small TCNQ crystals, Tien found that the resulting BLMs had voltammograms of similar shape but higher current than BLMs prepared from a clear solution [124]. Using a microscope they observed crystals, which were assumed to span the entire thickness of the BLM, providing conductance pathways across the membrane. In our experiments, the small crystals of TCNQ sometimes present in the BLM-forming solution applied to the troughs usually floated on the surface and were removed to assist formation and avoid disruption of formed membranes. On one or two occasions TCNQ crystals were observed on the periphery of a BLM and voltammograms had a higher current than when they were not present.

Iodine-modified membranes often produced considerable currents with the voltammograms sometimes approaching reversible systems. There appeared to be some leakage of iodine through the BLM, which often complicated the interpretation.

The application of cyclic voltammetry to membranes is still a relatively new field and the work presented here has indicated the need for more investigations into these models for the electron transfer occurring in biological membranes. The technique is certainly suitable for studies of this type on the membranes prepared here.

It has been suggested [125] that the peak height and peak position could be dependent on the lipid content of the BLMs and this would be an interesting aspect to investigate further.

One of the most interesting observations involves the shapes of voltammograms obtained. Although the characteristic voltammogram features two current peaks, a positive one and a negative one, both of the same height, another type, which shows a large non-linear increase of current for linearly increasing applied potential, has often been observed. Perhaps these systems do not yield peaked voltammograms or if so, the peaks might occur beyond the potential sweep range [125]. For most membranes it was possible to scan to a potential at least 200 mV more positive or negative than the rest potential (where there was no current flow) before the membrane ruptured.

Many voltammograms exhibited currents that reached a plateau. This behaviour usually occurs in solutions where stirring is continuous so that the species reacting is not depleted in the vicinity of the electrode, but stirring was not undertaken during voltammetric measurements. Recent work by Bond and co-workers involving electron transfer at protein-graphite electrode interfaces [126] may offer a reason for these types of plots. They suggest that voltammograms not exhibiting peaks may be a result of radial diffusion rather than an indication of limited electron transfer. Their model assumes that mass transport to the electrode occurs by radial diffusion when the density of surface-active sites are low and by linear diffusion when there are sufficient active sites to cause overlap of the diffusion layers. When radial diffusion occurs it is possible to obtain irreversible voltammograms with fast electron transfer. TCNQ would be a suitable modifier for further studies of this aspect occurring in bilayer membranes .

APPENDIX I - Materials Used

ORGANIC SOLVENTS

Freshly distilled solvents were used in order to remove impurities and water. The removal of water is important as phospholipids have a strong affinity for water,and its presence in the BLM-forming solution may result in thick patches of lipid occurring in the bilayers [23]. Solvents were also applied to an Adam-Langmuir trough to test for the presence of surface-active contaminants. The main solvent used in the BLM-forming solutions, n-hexane (Sigma Chemical Co.), was doubly-distilled . Squalene, supplied as a clear, odourless solution ($C_{30}H_{50}$, M.W. = 410.74; Sigma Chemical Co., No. S-3626, 98-100%), was stored at -20° C and used without further purification.

LIPIDS

All lipids were obtained from Sigma Chemical Co. and used as supplied, without further purification.

1-Monooleoyl-rac-glycerol (Monoolein; glyceryl monooleate; $C_{17}H_{33}.CO.OCH_2.CH(OH).CH_2OH$, [C 18:1, cis 9], approx. 99%), Sigma No. M-7765. M.W. = 356.5 $gmol^{-1}$.

L-α-phosphatidyl-L-serine (PS, approx. 98% from bovine brain), Sigma No. P-7769. Assume M.W. = 800 $gmol^{-1}$.

L-α-phosphatidylethanolamine (L-α-cephalin; PE, approx. 98% from *E. coli*), Sigma No. P-3511. Assume M.W. = 800 $gmol^{-1}$.

L-α-phosphatidylcholine (PC, approx. 99% from egg yolk), Sigma No. P-4139. Assume M.W. = 800 $gmol^{-1}$.

Natural lecithin was obtained as unbleached 98% lecithin granules (Lowan Whole Foods) from a health food shop.

Cholesterol (Sigma Chemical Co., No. C-8253, M.W. = 386.7 $gmol^{-1}$) was used to prepare oxidised cholesterol, following the method described by Tien [15]. The octane was removed *in vacuo* from 1 cm^3 of the supernatant resulting and the oxidised cholesterol was redissolved in 2 cm^3 of n-hexane with a small amount of chloroform (0.2 cm^3), which was needed to fully dissolve the oxidised cholesterol.

All lipids were stored under argon at about 5° C.

ELECTROLYTE AND BUFFER SOLUTIONS

All solutions were prepared using millipore-filtered water (Milli-Q Reagent Water). Sodium chloride (NaCl, analytical grade, Univar, Ajax Chemicals) was roasted at 400° C for 5 hours to destroy any organic contaminants. Potassium chloride (KCl) and sodium acetate (CH_3COONa) were supplied by BDH Chemicals and acetic acid (CH_3COOH) from Ajax Chemicals.

Buffers with pH values ranging from 3.6 to 5.6 were prepared by combining different amounts of acetic acid and sodium acetate.

MEMBRANE MODIFIERS

7,7,8,8-Tetracyanoquinodimethane (TCNQ, M.W. = 204.2 $gmol^{-1}$) was obtained from Sigma Chemical Co. (No. T-1636).

Iodine (I_2, M.W. = 253.8 $gmol^{-1}$) was obtained from Sigma Chemical Co. (No. I-0385).

REDOX COUPLES

All redox couples were obtained from BDH Chemicals or May and Baker, with the exception of quinhydrone. Quinhydrone ($C_{12}H_{10}O_4$, M.W. = 218.2 $gmol^{-1}$; Selby Co.) was recrystallised from millipore-filtered

water at 70° C to ensure that the two components, quinone (OC_6H_4O) and hydroquinone (HOC_6H_4OH), were present in equimolar proportions. The product was dried at room temperature in a dessicator (with phosphorous pentoxide as the drying agent).

The electroactive species, such as periodate ions, ferro/ferri-cyanide ions, ascorbic acid, used in the measurements , were considered unlikely to penetrate the BLM.

During most experiments, N_2 was flushed through the system in order to minimise the effect of O_2 on the reactions taking place.

APPENDIX II - Statistical Evaluation of Data

TEST OF SIGNIFICANCE [127]

The population means are denoted by μ_1 and μ_2 and the population variances by σ_1^2 and σ_2^2. The data consist of two samples of observations $\{x_1, x_2, ..., x_n\}$ and $\{y_1, y_2, ..., y_m\}$ and the hypothesis to be tested is that $\mu_1 = \mu_2$. For the case considered here, the variances are known.

The best estimates of μ_1 and μ_2 that can be obtained from these data are x and y, respectively, the two sample means. If the null hypothesis that $\mu_1 = \mu_2$ is true then $x - y$ has a distribution with zero mean and a variance $\sigma_1^2/n + \sigma_2^2/m$, since the variance of the difference $x - y$ is the sum of the variances of x and y.

The statistic chosen to measure agreement between μ_1 and μ_2 is, therefore:

$$X = \frac{x - y}{(\sigma_1^2/n + \sigma_2^2/m)^{1/2}}$$

The distribution of X depends on the distributions of the variables x and y. If these are assumed to be normal the distribution of X is a standardised normal distribution $N(0,1)$.

Values of X varied from between 5 and 200.

APPENDIX III - Comparison of Capacitance Values

The following table lists the experimental conditions and resulting capacitance values for several systems investigated by other researchers.

N is the number of membranes formed.

Lipid	Teflon partition thickness (μm)	N	Capacitance ($nFcm^{-2}$)	Type of membrane	Ionic strength (Molar)	Hole diameter (mm)	Ref.
PE/PE	19.0	-	650±10	monolayer	1.0	-	[71]
	-	-	630±30	-	-	-	[128]
	-	-	680±10	-	-	-	[129]
PE/PS asymmetric	6.4	54	661±50	monolayer	-	-	[66]
	9.5	38	665±25				
	12.7	55	663±44				
	19.0	49	694±42				
	25.4	25	724±48				
PE/PS	12.0	15	750	monolayer	-	0.225	[43]
Lecithin (average of 18 carbon atoms)	12.5	13	721±21	monolayer	0.01-0.1	0.2-0.3	[27]
egg lecithin, bovine cardioplin, plant PI, glycerol dioleate, oleoyoyl acid phosphate	25	-	900±100	monolayer	0.01	0.226	[40]

Lipid	Teflon partition thickness (μm)	N	Capacitance ($nFcm^{-2}$)	Type of membrane	Ionic strength (Molar)	Hole diameter (mm)	Ref.
Monoglyceride (18:1) [monoolein]	19.0	26	750±30	-	1.0	-	[71]
Monoolein	-	-	790±10	-	-	-	[129]
Monoolein	20.0	-	852±43	-	-	0.3	[130]
Monoolein (18:1)	12.5	33	745±24	monolayer	0.01-0.1	0.2-0.3	[27]
Monoolein	-	-	735	Solvent freeze-out	-	-	[32]
Monoolein	-	-	790±1	Solvent freeze-out	-	-	[32]
Monoolein	-	-	777±5	Solvent exclusion	0.1	-	[31]

REFERENCES

1. E. Overton: *Vjsehr. Naturf. Ges. Zürich* , 40 , 149, (1895).

2. J. F. Danielli and H. J. Davson: *J. Cellular Comp. Physiol.*, 5 , 483, (1934). ; J. F. Danielli and H. J. Davson: *J. Cellular Comp. Physiol.*, 5 , 495, (1934).

3. H. Davson and J. F. Danielli: "*The Permeability of Natural Membranes* ", University Press, (1952).

4. H. Fricke: *Phys. Rev.*, 21 , 708, (1923).

5. E. Gorter and F. Grendel: *J. Exptl. Med.*, 41 , 439, (1925).

6. J. D. Robertson: *Progr. Biophys. Biophys. Chem.*, 10 , 343, (1960).

7. J. F. Danielli: *J. Cell Comp. Physiol.*, 7 , 393, (1936).

8. I. Langmuir and D. F. Waugh: *J. Gen. Physiol.*, 21 , 745-755, (1938).

9. R. B. Dean, H. J. Curtis and K. S. Cole: *Science* , 91 , 50, (1940).

10. P. Mueller, D. O. Rudin, H. T. Tien and W. C. Wescott: "Symposium on the Plasma Membrane ", New York City, December, 1961. *Circulation* , 26 , 1167, (1962).

11. P. Mueller, D. O. Rudin, H. Ti Tien and W. C. Wescott: *Nature* , *London* , 194 , 979, (1962).

12. P. Mueller, D. O. Rudin, H. Ti Tien and W. C. Wescott: *J. Phys. Chem.*, 67 , 534, (1963).

13. P. Mueller and D. O. Rudin: *J. Theoret. Biol.*, 4 , 268, (1963).

14. M. Montal: *Methods in Enzymology* , 32 , 545-554, (1974).

15. H. Ti Tien: "*Bilayer Lipid Membranes (BLM): Theory and Practice* ", Marcel Dekker, Inc., N.Y., (1974).

16. P. Mueller, D. O. Rudin, H. Ti Tien and W. C. Wescott: in "*Recent Progress in Surface Science* ", Vol. 1, pg. 379, (J. F. Danielli, K. G. A. Pankhurst and A. C. Riddiford, eds.), Academic Press, Inc., New York, N.Y., (1964).

17. H. T. Tien: *J. Gen. Physiol.*, 52 , 1255, (1968).

18. Stephen H. White: *Biophys. J.*, 12 , 432-445, (1972).

19. A. Goldup, S. Ohki and J. F. Danielli: "*Recent Progress in Surface Science* ", 3 , 193-260, (1970).

20. M. K. Jain: "*The Bimolecular Lipid Membrane: A System* ", Van Nostrand Rheinhold Co., N.Y., (1972).

21. Alan Finkelstein: in *Methods in Enzymology* , 32 , 489-501, (1974).

22. Thomas E. Andreoli: in *Methods in Enzymology* , 32 , 513-539, (1974).

23. R. Fettiplace, L. G. M. Gordon, S. B. Hladky, J. Requena, H. P. Zingsheim and D. A. Haydon: *Methods Membrane Biol.* , 4 , 1-75, (1975).

24. F. A. Henn and T. E. Thompson: *J. Mol. Biol.*, 31 , 227-235, (1968).

25. S. H. White and T. E. Thompson: *Biochim. Biophys. Acta* , 323 , 7-22, (1973).

26. S. H. White: *Ann. N.Y. Acad. Sci.*, 303 , 243-265, (1977).

27. R. Benz, O. Fröhlich, P. Läuger and M. Montal: *Biochim. Biophys. Acta* , 394 , 323-334, (1975).

28. R. Benz and K. Tanko: *Biochim. Biophys. Acta* , 455 , 721-738, (1976).

29. T. P. Dilger, S. G. A. McLaughlin, T. J. McIntosh and S. A. Simon: *Science* , 206 , 1196-1198, (1979).

30. T. J. McIntosh, S. A. Simon and R. C. MacDonald: *Biochim. Biophys. Acta* , 597 , 445-463, (1980).

31. S. H. White: *Biophys. J.*, 23 , 337-347, (1978).

32. S. H. White: *Biochim. Biophys. Acta* , 356 , 8-16, (1974).

33. S. H. White: *Biophys.J.*, 15 , 95-117, (1975).

34.R. C. Waldbillig and G. Szabo: *Biochim. Biophys. Acta* , 557 , 295-305, (1979).

35. J. A. F. Kamp: *Annu. Rev. Biochem.*, 48 , 47-71, (1979).

36. G. P. Miljanich, P. P. Nemes, D. L. White and E. A. Dratz: *J. Membr. Biol.*, 60 , 249-255, (1981).

37. M. D. Houslay and K. K. Stanley: "*Dynamics of Biological Membranes* ", Wiley, Toronto, (1982).

38. J. A. Higgins and C. A. Pigott: *Biochim. Biophys. Acta* , 693 , 151-158, (1982).

39. D. M. Michaelson, G. Barkai and Y. Barenholz: *Biochem. J.*, 211 , 155-162, (1983).

40. M. Montal and P. Mueller: *Proc. Nat. Acad. Sci. USA* , 69(12), 3561-3566, (1972).

41. M. Takagi, K Azuma and U. Koshimoto: *Ann. Report Biol. Works Fac. Sci. Osaka Univ.* , 13 , 107, (1965).

42. S. M. White: *Science* , 207 , 1075, (1980).

43. Pierre Tancrède, Paul Paquin, André Houle and Roger M. LeBlanc: *J. Biochem. Biophys. Methods* , 7 , 299-310, (1983).

44. R. Laprade and J. -Y. Lapointe: *Rev. Can. Biol. Expt.*, 41 , 13, (1982).

45. Stephen H. White, Daniel C. Petersen, Sidney Simon and Masuo Yafuso: *Biophys. J.*, 16 , 481-489, (1976).

46. Sylvain Robert, Pierre Tancrède, André Houle and Roger M. LeBlanc: *Photochem. Photobiol.*, 41(1), 101-106, (1985).

47. R. Benz, F. Beckers and U. Zimmermann: *J. Membr. Biol.*, 48 , 181, (1979).

48. V. B. Arakelyan, H. R. Hachatryan and N. S. Matinyan: *Stud. Biophys.*, 93 , 69, (1983).

49. Philip L. Yeagle: *Biochim. Biophys. Acta* , 822 , 267-287, (1985).

50. Paul A. Hyslop, Benoit Morel and Richard D. Sauerheber: *Biochemistry* , 29 , 1025-1038, (1990).

51. John Hjort Ipsen, Ole G. Mouritsen and Myer Bloom: *Biophys. J.* , 57 , 405-412, (1990).

52. Phillip L. Yeagle, Arlene D. Albert, Kathleen Boesze-Battaglia, Joyce Young and James Frye: *Biophys. J.* , 57 , 413-424, (1990).

53. G. W. Stockton and I. C. P. Smith: *Chem. Phys. Lipids*, 17 , 251-263, (1976).

54. R. Jacobs and E. Oldfield: *Biochemistry* , 18 , 3280-3285, (1979).

55. M. Y. El-Sayed, T. A. Guion and M. D. Fayer: *Biochemistry* , 25 , 4825-4832, (1986).

56. C. Huang, L. Wheeldon and T. E. Thompson: *J. Mol. Biol.*, 8 , 148, (1964).

57. T. Hanai, D. A. Haydon and J. Taylor: *J. Theoret. Biol.*, 9 , 422, (1965).

58. T. Hanai, D. A. Haydon and J. Taylor: *J. Theoret. Biol.*, 9 , 433, (1965).

59. G. M. Barrow: "*Physical Chemistry* ", McGraw-Hill, (1961). ; W. J. Moore: "*Physical Chemistry* ", Prentice Hall, (1961).

60. When a solution of chloroform (b.p. 61.2° C) and methanol (b.p. 64.7° C) is distilled at 53.4° C the solution coming off will be 12.6% methanol (Rubber Handbook of Chemistry and Physics, CRC Press). After this solution was used to clean the Teflon troughs, it was distilled and used again.

61. Pierre Tancrède: Private Communication.

62. H. V. Malmstadt, C. G. Enke and E. C. Toren: "*Electronics for Scientists* ", pg. 573, W. A. Benjamin, Inc. New York, (1963).

63. The voltage applied to the unknown during the measurement is approximately as follows : Ranges 1 and 2 - 25 mV rms, Ranges 3 and 4 - 250 mV rms, Ranges 5 and 6 - 2.5 V rms and Range 7 - 25 V rms.

64. T. Hanai, D. A. Haydon and Janet Taylor: *Proc. Roy. Soc. A* , 281 , 377-391, (1964).

65. William A. Huemoeller and H. Ti Tien: *J. Chem. Education* , 47(6), 469-470, (1970).

66. Marc Brullemans and Pierre Tancrède: *Biophys. Chem.* , 27 , 225-231, (1987).

67. J. R. Smith, H. G. L. Coster and D. R. Laver: *Biochim. Biophys. Acta* , 812 , 181, (1985).

68. Vitaly Vodyanoy and Randall B. Murphy: *Biochim. Biophys. Acta* , 687 , 189-194, (1982).

69. A. V. Babakov, L. N. Ermishkin and E. A. Liberman: *Nature (London)* , 210 , 953-955, (1966).

70. Stephen H. White: *Biophys. J.*, 10 , 1127-1148, (1970).

71. Osvaldo Alvarez and Ramon Latorre: *Biophys. J.*, 21 , 1-17, (1978).

72. B. E. Cooper: "Statistics for Experimentalists", Chapter 6, Pergamon Press, London, (1969).

73. H. Ti Tien and A. Louise Diana: *Chem. Phys. Lipids* , 2 , 55-101, (1968).

74. Shinpei Ohki: *Biophys.J.*, 9 , 1195-1205, (1969).

75. T. J. McIntosh: *Biochim. Biophys. Acta* , 513 , 43-58, (1978).

76. K. S. Cole and H. J. Curtis: in "*Medical Physics.(11)* ", (O. Glasser, ed.), pg. 83, Year Book Publisher, (1950).

77. H. Pauly and L. Packer: *J. Biophys. Biochem. Cytol.*, 7 , 603, (1960). ; W. F. Floyd: *Sci. Progr. (London)* , 36 , 214, (1948).

78. L. de Bernard: *Bull. Soc. Chim. Biol.*, 40 , 161, (1958). ; J. L. Taylor:*Thesis* , Cambridge.

79. Lund: *J. Exptl. Zool.*, 51 , 265, (1928).

80. A. Szent-Gyorgyi: *Science* , 93 , 609, (1941).

81. R. P. F. Gregory: "*Biochemistry of Photosynthesis* ", pgs. 126-165, Wiley, New York, (1978).

82. D. E. Metzler: "*Biochemistry : The Chemical Reactions of Living Cells* ", Chap. 5, Academic Press, New York, (1977).

83. A. C. Becquerel: *Compt. Rend. H* , 64 , 619-624, (1867).

84. G. Braun: *Wied. Ann.*, 44 , 473-495, (1891).

85. P. Mueller, D. O. Rudin, H. T. Tien and W. C. Wescott: *Circulation* , 26 , 1167, (1962).

86. R. Antolini, A. Gliozzi and A. Gorio (Eds.): "*Transport in Biomembranes : Model Systems and Reconstitution* ", Raven Press, New York, (1982).

87. F. T. Hong: in "*Biochemistry: Ions, Surfaces, Membranes* ", (M. Blank, ed.), *Adv. Chem. Ser.*, No. 188, pgs. 211-237, A. C. S., Washington, DC, (1980).

88. A. Hattenbach, J. Gundel, G. Hermann, D. Haroske and E. Muller: *Biochem. Physiol. Pflanz.*, 177 , 611, (1982).

89. E. Bienvenue, P. Seta, A. Hofmanova, C. Gavach and M. Momenteau: *J. Electroanal. Chem.*, 162 , 275, (1984).

90. B. Karvaly and H. Pant: *Studia Biophys.*, 33 , 51-58, (1972).

91. J. Heubner: *Biochim. Biophys. Acta* , 406 , 178-186, (1975).

92. M. Mangel: *Biochim. Biophys. Acta* , 419 , 404-410, (1976)

93. H. Ti Tien: *Progress in Surface Science* , 30(1/2), 1-199, (1989).

94. H. Ti Tien, Bela Karvaly and Paul K. Shieh: *J. Coll. Interface Science* , 62(1), 185-188, (1977).

95. H. Ti Tien and Z K. Lojewska: *Biochem. Biophys. Res. Comm.*, 119(1) , 372-375, (1984).

96. H. Ti Tien: *J. Phys. Chem.*, 88 , 3172-3174, (1984)

97. Richard S. Nicholson and Irving Shain: *Anal. Chem.*, 36(4), 706-723, (1964).

98. Richard S. Nicholson and Irving Shain: *Anal. Chem.,* 37(2), 190-195, (1965).

99. Richard S. Nicholson: *Anal. Chem.,* 37(6), 667-671, (1965).

100. Richard S. Nicholson: *Anal. Chem.,* 37(11), 1351-1355, (1965).

101. Allen J. Bard and Larry R. Faulkner: "*Electrochemical Methods : Fundamentals and Applications* ", John Wiley and Sons, New York, (1980).

102. Arnold Weissberger and Bryant W. Rossiter (eds.): "*Techniques of Chemistry : Vol. I, Physical Methods of Chemistry* ", Wiley-Interscience, New York, (1971).

103. J. T. Maloy: *J. Chem. Educ.*, 60(4), 285-289, (1983).

104. Dennis H. Evans, Kathleen M. O'Connell, Ralph A. Petersen and Michael J. Kelly: *J. Chem. Educ.*, 60(4), 290-292, (1983).

105. Pawel Krysinski and H. Ti Tien: *Bioelectrochem. Bioenerg.*, 16 , 185-191, (1986).

106. H. Ti Tien: *Bioelectrochem. Bioenerg.*, 15 , 19-38, (1986).

107. D. Acker and W. R. Hertler: *J. Am. Chem. Soc.*, 84 , 3370-3374, (1962). L. R. Melby, *et al.* : *J. Am. Chem. Soc.*, 84 , 3374-3387, (1962). L. R. Melby: *Can. J. Chem.*, 43 , 1448-1453, (1965). O. H. LeBlanc: in "*Physics and Chemistry of the Organic Solid State* ", pg. 182, Interscience, (1967).

108. Martin R. Bryce and Lynne C. Murphy: *Nature* , 309 , 119-126, (1984).

109. Rosenberg and Jendrasiak: *Chem. Phys. Lipids*, 2 , 47, (1968).

110. B. Rosenberg and B. Bhowmik: *Chem. Phys. Lipids*, 3 , 109, (1969).

111. P. Läuger, J. Richter and W. Lesslauer: *Ber. Bunsenges. Phys. Chem.*, 71 , 906, (1967).

112. B. Bhowmik, G. L. Jendrasiak and B. Rosenberg: *Nature*, 215 , 842, (1967).

113. A. Finkelstein and A. Cass: *J. Gen. Physiol.*, 52 , 145, (1968).

114. Mahendra K. Jain , Alfred Strickholm, Frederick P. White and E. H. Cordes: *Nature* , 227 , 705-707, (1970).

115. E. A. Liberman, V. P. Topaly, L. M. Tsofina and A. M. Schrob: *Biofysika* , 14 , 56, (1969).

116. Benoy B. Bhowmik, Ruma Dutta and Papiya Nandy: *J. Colloid and Interface Science*, 122(2), 450-455, (1988).

117. F. Wudl, G. M. Smith and E. J. Hufnagel: *JCS Chem. Comm.*, 1453-1454, (1970).

118. Handbook of Chemistry and Physics, CRC Press (Chemical Rubber Co.)

119. H. Ti Tien: *Bioelectrochem. Bioenerg.*, 13 , 299-316, (1984).

120. H. Ti Tien: *J. Phys. Chem.*, 88(15), 3172-3174, (1984).

121. Felix T. Hong: *Photochem. Photobiol.* , 24 , 155-189, (1976).

122. Christopher J. Bender and H. Ti Tien: *J. Electroanal. Chem.*, 284 , 217-227, (1990).

123. Christopher J. Bender and H. Ti Tien: *Anal. Chim. Acta* , 198 , 55, (1987).

124. Pawel Krysinski and H. Ti Tien: *Bioelectrochem. Bioenerg.*, 19 , 227-233, (1988).

125. Jan Kutnik and H. Ti Tien: *Bioelectrochem. Bioenerg.* , 16 , 435-447, (1986).

126. F. A. Armstrong, A. M. Bond, H. Allen O. Hill, B. Nigel Oliver ans Ioanna S. M. Psalti: *J. Am. Chem. Soc.*, 111 , 9185-9189, (1989).

127. D. D. Perrin and Boyd Dempsey: "*Buffers for pH and Metal Ion Control* ", Chapman and Hall, London, 1974.

128. L. Ebihara and J. E. Hall: *Biophys. J.*, 28 , 185, (1979).

129. J. Reyes and R. Latorre: *Biophys. J.*,28 , 259, (1979).

130. I. Vodyanoy and J. E. Hall: *Biophys. J.*, 46 , 187, (1987).

Effect of 2.45–GHz–Microwave Radiation on Permeability of Unilamellar Liposomes to 5(6)–Carboxyfluorescein. Evidence of Non–Thermal Leakage.

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SUMMARY

The influence of 2.45 GHz microwave radiation on the membrane permeability of unilamellar liposomes was studied using the marker 5(6)–carboxyfluorescein trapped in phosphatidylcholine liposomes.

The release of the fluorescent marker was followed by spectrofluorimetry after an exposure of 10 minutes to either microwave radiation or to heat alone of the liposome solutions. A significant increase of the permeability of carboxyfluorescein through the membrane was observed for the microwave–exposed samples compared to those exposed to normal heating only. Exposure to 2.45 GHz microwave radiation of liposomes has been previously found to produce increased membrane permeability as compared with heating. However, in contrast to previous studies, the observations reported here were made above the phase transition temperature of the lipid membrane.

The experimental setup included monitoring of the temperature during microwave exposure simultaneously at several points in the solution volume using a fiberoptic thermometer.

Possible mechanisms to explain the observations are discussed.

INTRODUCTION

It is well known that leakage from the interior of liposomes and natural membranes of entrapped substances may take place [1–7]. More recently, an increase in such leakage upon microwave exposure of these systems has been reported [8–13], although the observation of the effect is not unequivocal [14,15]. In the work to be described here we studied the release of the fluorescent marker 5(6)–carboxyfluorescein from liposomes made from L–α–phosphatidylcholine. Particular attention has been directed towards ensuring that the liposomes were of uniform size, of narrow size–distribution and possessing pure lipid membranes. On the basis of our results we confirm that microwave exposure enhances the release of the dye. Although no definite mechanism for the effect emerges we shall be able to exclude some potential mechanisms for the phenomena observed.

MATERIALS AND METHODS

Preparation of unilamellar liposomes

The lipid used was L–α–phosphatidylcholine (PC) from soybean in chloroform solution (SIGMA Cat.no. P6263) and the dye to be entrapped was 5(6)–carboxyfluorescein (CF) also obtained from SIGMA (Cat.no. C 7153). The method involves adding the dye during the liposome preparation process which results in its incorporation into the liposome interior. The complete preparation is carried out in two major steps. First multilamellar liposomes are produced which may be freeze–dried and stored ready for the next stage of the preparation of unilamellar liposomes through extrusion. The preparative procedures are described in more detail as follows.

250 mg of PC in chloroform solution was evaporated to dryness in a rotary evaporator and the dry PC film redissolved in first 2 cm^3 and then an additional 8 cm^3 of a 0.017 mol dm^{-3} CF solution in 0.05 mol dm^{-3} tris/saline buffer of pH 7.4. In order to assist dispersion of the PC a bath sonicator (Branson Sonifier B–12) at a power setting near 65 W was used. Vortexing of the solution produced multilamellar vesicles which were then 7–8 times in succession frozen in liquid nitrogen and thawed in order to ensure equal distribution of the solute between lamellae and adequate hydration of the lipid. Finally, the liposome solutions were freeze–dried and stored until the next stage of the preparative procedure; we note that freeze–drying has been reported to enhance the capacity of encapsulating solutes by the liposomes (7).

The preparation of the unilamellar liposomes proceeded from the freeze–dried PC/CF mixture; this was first rehydrated by dissolving it in 10 cm^3 of 0.05 mol dm^{-3} tris/saline buffer. The solution was then passed through an extruder (Lipex Biomembranes Inc., Vancouver, Canada) equipped with a base filter and a 0.1 μm filter (Nucleopore, SN:110605). The solution was extruded 5 times interspersed with freezing/thawing using liquid nitrogen as for the preparation of the multilamellar liposomes. Finally the excess CF was separated from the liposomes by gel–filtration using columns packed with Sephadex G–25. The resulting unilamellar liposomes are stable for at least 2–3 days at a storage temperature of 5^0C; however, all experiments were carried out as soon as possible, usually within 10 hours, after completion of the preparation.

The phase transition temperature of the liposomes was found to be within the reported range of between -15^0C and $+7^0$C of phosphatidylcholine liposomes (16). The size and polydispersity of the liposomes were determined by dynamic light scattering using an instrument consisting of a laser light source, a temperature–controlled cell

holder and a MALVERN K7027 'LOGLIN' correlator. The apparatus was calibrated using a standard monodisperse sample of polystyrene latex spheres.

Spectrophotometric measurements

Absorbance and fluorescence of the solutions were measured using a Varian Cary 219 spectrophotometer and an Aminco SPF–500 spectrofluorimeter, respectively. For the purposes of the fluorescence measurement the liposome solutions were diluted with tris/saline buffer to an absorbance near 0.25 in a 0.5 cm cuvette at 490 nm, the excitation wavelength to be used. Fluorescence emission was measured at 518 nm.

The liposomes were checked for the possibility of the leakage of the dye through the membrane by measuring the fluorescence intensity immediately after dilution of the liposome sample and comparing it with that measured 3 hours later. Only sample solution that showed no change in fluorescence intensity were used for the subsequent experiments of microwave exposure.

All spectrofluorimetry was carried out at constant room temperature of 21°C; however, between measurements the samples were placed in an ice–bath and kept in the dark.

Microwave exposure experiments

Microwave radiation was generated by a continuous wave magnetron operating at 2.45 GHz. The magnetron was coupled to an IEC R32 waveguide (S–band) which was terminated with a horn antenna with aperture dimensions of 157 mm x 135 mm in the E and H planes, respectively. The output power was monitored with a power meter

connected to a waveguide directional coupler situated between the magnetron and the antenna. All irradiations were performed at a distance of 0.4 m between the sample and the antenna aperture. The walls of the exposure room were lined with microwave absorbing material (Eccosorb, Emerson and Cumming Inc., Canton, MA, USA).

The sample container was made of polytetrafluorethylene (Teflon^{R}) with a volume of 1 cm^3; it has been described in detail elsewhere (17). The sample container was placed inside a poly(methylmethacrylate) (Perspex^{R}) trough, as shown in Figure 1, through which water was circulated from a thermostat placed well outside the radiation field. Stirring of the sample itself was achieved by a slow flow of air bubbles *via* a small plastic tube protruding through the lid of the sample container. Temperature of the sample was measured using a Luxtron 755 multichannel fluoroptic thermometer with four fiberoptic non–perturbing probes positioned at different depths in the sample. The thermometer and the power meter were connected to an HP 85 desk computer based measurement system programmed to register the temperature on all four probes every fourth second. We ascertained that the presence of the thermometer had no influence on the fluorescence results.

Experimental procedure

The experimental procedure was as follows. Liposome samples were removed from ice, left at room temperature for one minute and then placed in a waterbath at 38.5^{0}C for another minute. One liposome sample (1 cm^3) was exposed to microwaves for 10 minutes and another identical sample was placed, without exposure to radiation, in a water bath kept at a temperature 1.0 – 1.5^{0}C higher than the final temperature of the microwave irradiated sample; the heating of this second sample was

continued for 11 minutes compensating for the time taken to fill the sample container for microwave exposure. After the exposure period the temperature of both samples was decreased to 10°C rapidly, within about 30 seconds, the samples were then transferred to 0.5 cm^3 silica cuvettes and their fluorescence determined as soon as they warmed to room temperature of 21°C. Finally, the average liposome size of the samples was measured by dynamic light scattering and compared with that prior to the microwave or heat exposure.

Dosimetry

Specific absorption rate (SAR) of the radiation was determined in a separate experiment. The test solution in the sample container and the stationary water in the Perspex^R trough were allowed to come to room temperature; without bubbling air through the sample the container was exposed to microwave radiation for about 10 minutes and the temperature of the sample was recorded as a function of time of exposure. If we write w for the absorbed power and M for the mass then SAR, the absorbed power per unit mass is given by

$$SAR = \frac{w}{M} = C_p \cdot \frac{\Delta T}{\Delta T} \qquad (1)$$

where C_p is the heat capacity and $\Delta T/\Delta T$ corresponds to the initial slope of the temperature versus time function during irradiation.

Determination of Complex permittivity

The complex permittivity is defined as

$$\epsilon^* = \epsilon' - j\epsilon'' \qquad (2)$$

where ϵ' is the real permittivity, ϵ'' is the loss factor and $j^2 = -1$. The complex permittivity of solutions of liposomes at a temperature of 21°C and at various concentrations was determined at 3.1 GHz using the resonance method. The resonance curves were displayed and measured by means of a network analyzer connected to the cavity; the real permittivity is determined by the frequency of the resonance, *f*, and the loss by the sharpness of the resonance. The latter is expressed as the Q–factor,

$$Q = \Delta f / f \qquad (3)$$

where Δf is the bandwidth at an attenuation of 3 decibels of the maximum current (18,19). The relevant formulae for a thin sample in a cylindrical TM_{010} cavity are given by (19).

$$\frac{\epsilon'}{\epsilon_0} = 1 + 0.539 \frac{V}{v} \frac{(f_0 - f_1)}{f_0} \qquad (4)$$

$$\frac{\epsilon''}{\epsilon_0} = 0.269 \frac{V}{v} \left[\frac{1}{Q_1} - \frac{1}{Q_0}\right] \qquad (5)$$

where v is the volume of the sample, V the volume of the cavity and the subscripts 1 and 0 refer to the results of the measurements with and without the sample in the cavity, respectively.

RESULTS

From the dynamic light scattering measurements we obtained the average diameter of 185 ± 10 nm of the liposomes in the samples used. The standard deviation was estimated from an essentially monoexponential

autocorrelation function (20). Its value reflects low polydispersity of the liposomes in the preparations. Importantly, the same measurements showed no significant change in either the average size of the liposomes or its standard deviation for either the microwave irradiated samples or the control samples exposed to heat only.

In Figure 2 are shown the results of the fiberoptic temperature measurements for both the microwave–exposed and the normally heated control samples. As already referred to in the previous section the temperature to which the control samples were exposed was somewhat in excess of that reached by the irradiated samples as a result of the absorption of microwave radiation. This was done to ensure that the observed effect could not be due to a macroscopic overheating of the microwave–exposed sample.

In the dosimetry determination the temperature increase as a function of time was registered when the liposome sample was exposed to high intensity microwave radiation. From the initial slopes of these curves, using, as an approximation, the heat capacity value of water at 20^{0}C and scaling to the output power used we obtain SAR values of 38 $\pm$ 1 W/kg for the liposome solution.

In order to determine the microwave dissipation of the liposome solution the complex permittivity ϵ was mesured as a function of liposome concentration, see Figure 3. The imaginary part of ϵ, which is proportional to the microwave losses, increases with liposome concentration.

In figure 4 are shown the fluorescence spectra of four liposome solutions. The minimum fluorescence intensity is shown by a sample before exposure to either radiation or heat and the maximum intensity is achieved when the sample is heated to 90^{0}C when, due to rupture of the liposomes, all CF is released into free solution. The significant result is shown by the remaining two spectra: the leakage of the dye as a result of microwave

irradiation is greater (58% of maximal fluorescence) compared with the heated control (36% of maximal fluorescence) in spite of the fact that the control was exposed to higher temperatures.

As an additional control experiment we observed that if a sample was heated to 45°C, instead of 40°C, the leakage of the dye increased by a further 7%. This would give a leakage of 43% of maximal release for the normally heated sample, which is still significantly less than the 58% resulting from microwave irradiation.

DISCUSSION

We first note that the method of preparation of the liposomes did not involve organic solvents, detergent and, except for aiding the dissolution of the *lipids*, the use of sonication; all of these are factors that make liposomes more complex and may affect their stability. In addition, the average size of the liposomes was reproducible from sample to sample showing relatively little polydispersity. Thus the quality of the liposome preparations adds confidence to the assertion that the results of microwave exposure experiments reflect a true property of the lipid bilayer forming the vesicle membranes.

Next we recall that we studied the liposomes, which were kinetically stable, well above their liquid–crystalline phase transition temperature. Liposomes are generally known to release encapsulated water soluble contents more readily near to their phase transition temperature (1–7,11,13). Our results extend this observation by demonstrating that liposomes display leakage that increases with temperature far above their lipid membrane phase transition temperature.

It has also been proposed (13) that increased liposome leakage as a result of microwave exposure should occur *only* near the phase transition

temperature. However, the proposition that such changes in permeability are associated with the lipid liquid–crystalline transition are not substantiated by our results that recorded a significant increase in leakage of liposomes above their phase transition temperature upon exposure to microwave radiation compared with the heated control samples.

The mechanism through which microwave radiation causes an increase in the membrane permeability is, of course, of prime interest and as a result of this study we may, at least, exclude some potential mechanisms.

First, the release of the dye on microwave radiation increases without a corresponding release of membrane phospholipids as the average size of the liposomes remains unchanged as measured by dynamic light scattering. We conclude, in agreement with a previous report (13), that the increase in permeability cannot be due to the disruption of the membranes to any significant extent.

Second, it is unlikely that the release of the dye from the liposome interior is due to dielectric breakdown of the phospholipid bilayer; pore formation has been reported to occur as a result of intense electric field exposure when the field strength exceeded 3000 kV m^{-1} (21), several orders of magnitude larger than the electric field strength of approximately 10 V m^{-1} used in this study.

Third, thermal effects deserve most serious consideration. We believe that since the samples were stirred the possibility of local temperature rises due to inhomogeneous heating may be excluded. There is, however, a significantly larger dissipation of microwave energy in the liposome solution compared with buffer and, accordingly, local heat effects due to inhomogeneous *absorption* of the radiation must be considered. We assume that in this relatively dilute liposome solution the structure of the solvent remains sensibly unchanged and attribute the increased microwave

energy dissipation to the liposomes present. The largest localized heating effect would occur if the energy was dissipated within the membrane rather than in the whole of the liposome cavity leading to a possible membrane disruption. However, dynamic light scattering does not show the presence of debris from ruptured liposomes after microwave irradiation; indeed, as shown in the Appendix, the diffusion of heat from the thin membrane to the surrounding is highly efficient and should prevent local temperature rises of sufficient magnitude to cause disruption of the membrane.

The increase in the leakage of the dye from the liposomes with increasing temperature without disruption of the membrane in the absence of microwave radiation implies that, at least for the particular liposomes studied here, thermal motion of the lipid molecules within the membrane results in the formation of transient pores through which entrapped molecules may be released from the interior of the liposomes. Thus, although we excluded the possibility of a disruption of the liposome membrane, localized energy dissipation of microwave radiation may, nevertheless, cause the formation of additional transient pores that may account for the release of the entrapped dye in excess of the leakage from the non–irradiated control samples. Accepting this explanation for our observations leads to the question of why additional energy dissipation should occur in the liposome membranes, particularly in view of the fact that pure lipid systems generally show much lower absorption of microwaves than water. Although we cannot provide at this stage an answer to the question posed, we believe that, as a consequence of the particular structure of the liposomes, microwave exposure may lead to localized absorption in the membrane.

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APPENDIX

The transfer rate of energy due to microwave absorption per unit volume of membranes, j in units of J s^{-1} m^{-3}, may be estimated from the additional temperature rise, ΔT, observed for liposome solution, compared to buffer solution, during the exposure time Δt:

$$j = (C_p \rho \Delta T / \Delta t) (V / v) \qquad (A1)$$

where C_p and ρ are, respectively, the average heat capacity and density of the solution, V is the volume of the solution and v that of the membrane. Assume first that there is no conduction of heat from the membrane to the ambient solution; the corresponding temperature rise in the membrane, ΔT_m, is then obtained from:

$$j \Delta t = \Delta T_m C_{pm} \rho_m \qquad (A2)$$

where C_{pm} and ρ_m are, respectively, the heat capacity and density of the membrane. This may represent a very substantial rise in temperature; for example, $\Delta T_m = 1000$ K is obtained for an observed bulk temperature rise of $\Delta T = 2$ K with the approximations $C_{pm} \simeq C_p$ and $\rho \simeq \rho_m$ when the lipid volume fraction is $v/V = 0.002$.

A membrane heated to such an extent is certainly expected to disintegrate; however, as evidenced by the dynamic light scattering results,

this does not happen which leads to the conclusion that owing to the small thickness of the membrane the diffusion of heat from it to the surrounding medium is highly efficient. This proposition may be tested by approximate numerical estimates of the cooling of the membrane. Specifically, according to the Fourier law of thermal diffusion the thermal energy flux, ϵ in units of J s^{-1} m^{-2}, is proportional to the temperature gradient in the system; if we assume that the maximum temperature rise occurs in the centre of the bilayer of total thickness d, the heat transfer rate at the membrane boundary per unit area is

$$\epsilon = -\lambda \, \Delta T_m / (0.5 \, d) \qquad (A3)$$

where we may take the value of $\lambda = 0.3$ J s^{-1} m^{-1} K^{-1} for glycerol to be a reasonable approximation for the lipids and d = 5.0 nm. One now defines a characteristic cooling time, τ, which is the time required to transfer through the outer membrane boundary into bulk solution the heat generated inside the membrane due to the absorption of radiation, calculated form the equality

$$\Delta t \; j \; A \; d = \tau \; \epsilon \; A \qquad (A4)$$

where A is the outer membrane surface area. Using the approximate values of $C_{pm} = 4200$ J kg^{-1} and $\rho_m = 1000$ kg m^{-3} one obtaines $\tau \simeq 10^{-10}$ s.

Next one needs some estimate of the time required for the disintegration of the membrane. This we take, tentatively, to be no less than the smallest relaxation time, the rotational correlation time, τ_{rot}, of a spherical membrane fragment whose radius, r, determined by the thickness of the membrane, is 2.5 nm. The Debye expression [22].

$$\tau_{rot} = 4\ \pi\ r^3\ \eta\ /\ (3\ k\ T) \qquad (A5)$$

requires a value for η, the viscosity of the medium. Taking the viscosity to be that of water one calculates $\tau_{rot} \simeq 10^{-8}$ s; this is the lower limit for τ_{rot} since the rest of the membrane, laterally joining the revolving part, must have a damping effect increasing the effective value of the viscosity. Nevertheless, the results of these numerical estimates show that the cooling time is at least 2 orders of magnitude less than the minimum time required for the membrane to disintegrate.

REFERENCES

1 Haest, C.W.M., de Gier, J., van Es, G.A., Verkleij, A.J., van Deenen, L.L.M. (1972) Biochim. Biophys. Acta 288, 43–53.

2 Papahadjopoulos, D., Jacobson, K., Nir, S., Isac, T. (1973) Biochem. Biophys. Acta 311, 330–348.

3 Inoue, K. (1974) Biochim. Biophys. Acta 339, 390–402.

4 Blok, M.C., van der Neut–Kok, E.C.M., van Deenen, L.L.M., de Gier, J. (1975) Biochim. Biophys. Acta 406, 187–196.

5 Blok, M.C., van Deenen, L.L.M., de Gier, J. (1976) Biochim. Biophys. Acta, 433, 1–12.

6 Magin, R.L., Niesman, M.R. (1984) Chem. Physics Lipids 34, 245–256.

7 Magin, R.L., Weinstein, J.N. (1984) In Gregoriadis G (ed) "Liposome Technology". Boca Raton. FL:CRC Press, Vol. 3 pp 137–155.

8 Olcerst, R.B., Belman, S., Eisenbud, M., Mumford, W.W., Rabinowitz, J.R. (1980) Radiat. Res. 82, 244–256.

9 Fisher, P.D., Poznansky, M.J., Voss, W.A.G. (1982) Radiat. Res. 92, 411–422.

10 Cleary, S.F., Garber, F., Liu, L.M. (1982) Bioelectromagnetics 3, 453–466.

11 Liburdy, R.P., Penn, A. (1984) Bioelectromagnetics 5, 283–291.

12 Liburdy, R.P., Vanek Jr, P.F. (1985) Radiat. Res. 102, 190–205.

13 Liburdy, R.P., Magin, R.L. (1985) Radiat. Res. 103, 266–275.

14 Rafferty, C.N., Knutson, J.R., Effects of pulsed microwave fields on soluble proteins and liposomes, IEEE/Ninth annual conference of the engineering in medicine and biology society–0701.

15 Liu, Li–Ming, Cleary, S.F. (1988) Bioelectromagnetics, 9, 249–257.

16 Schmidt, K.H., Liposomes as drug carriers, Symposium Tübingen, October 1984, Georg Thieme Verlag, Stuttgart, New York, (Hässle AB).

17 Hamnerius, Y. (1983) Hereditas, 98, 43–59.

18 Vaughan, W.A., Smyth, C.P., Powles, J.G. (1972) in Weissberger, A. and rossiter, B.W. (Eds.), "Physical methods of chemistry" (Wiley–Interscience).

19 Westphal, W.B. (1954) in von Hippel, A.R. (Ed), "Dielectric materials and applications" (John Wiley & Sons), p. 70, pp. 121–122.

20 Koppel, D.E. (1972) J. Chem. Phys., 57, 4814–4820.

21 Teissie, J., Tsong, T.Y. (1981) Biochemistry 20, 1548–1554.

22 Atkins, P.W. (1986) (Ed), "Physical Chemistry" (3rd edition, Oxford University Press) p. 621.

Figure legends

Fig. 1 Schematic picture of PTFE (Teflon) exposure cuvette (A). The cuvette is lowered into a water jacket (B), which is connected to a thermostated water bath by tubing (C). The lid (D) has holes (E) for optical fibre temperature probes. The sample is stirred by air, which is supplied via the tube (F).

Fig. 2 Temperature as a function of time in a representative 2.45–GHz microwave–exposed sample (A) with temperature measurements in 4 points taken with a fiberoptic thermometer. Curves (B) shows temperature measurements in the control sample which is in the water bath.

Fig. 3 Results of measurements of the complex permittivity as a function of the lipid concentration in tris/saline buffer.

a, the real part of the complex permittivity

b, the imaginary part of the complex permittivity

The error bars indicate standard error of mean.

Fig. 4 Representative fluorescence, emission, spectra obtained on four differently treated liposome samples in a 0.5 cm quarts cuvette:

(– – –) microwave–exposed (2.45 GHz) sample (38°C, 10 minutes), (· · ·) normally heated sample (39°C, 11 minutes), (——) non–exposed sample and (–·–·–) sample fluorescence after total disintegration of liposomes (90°C, 10 minutes) defines the upper limit of membrane leakage.

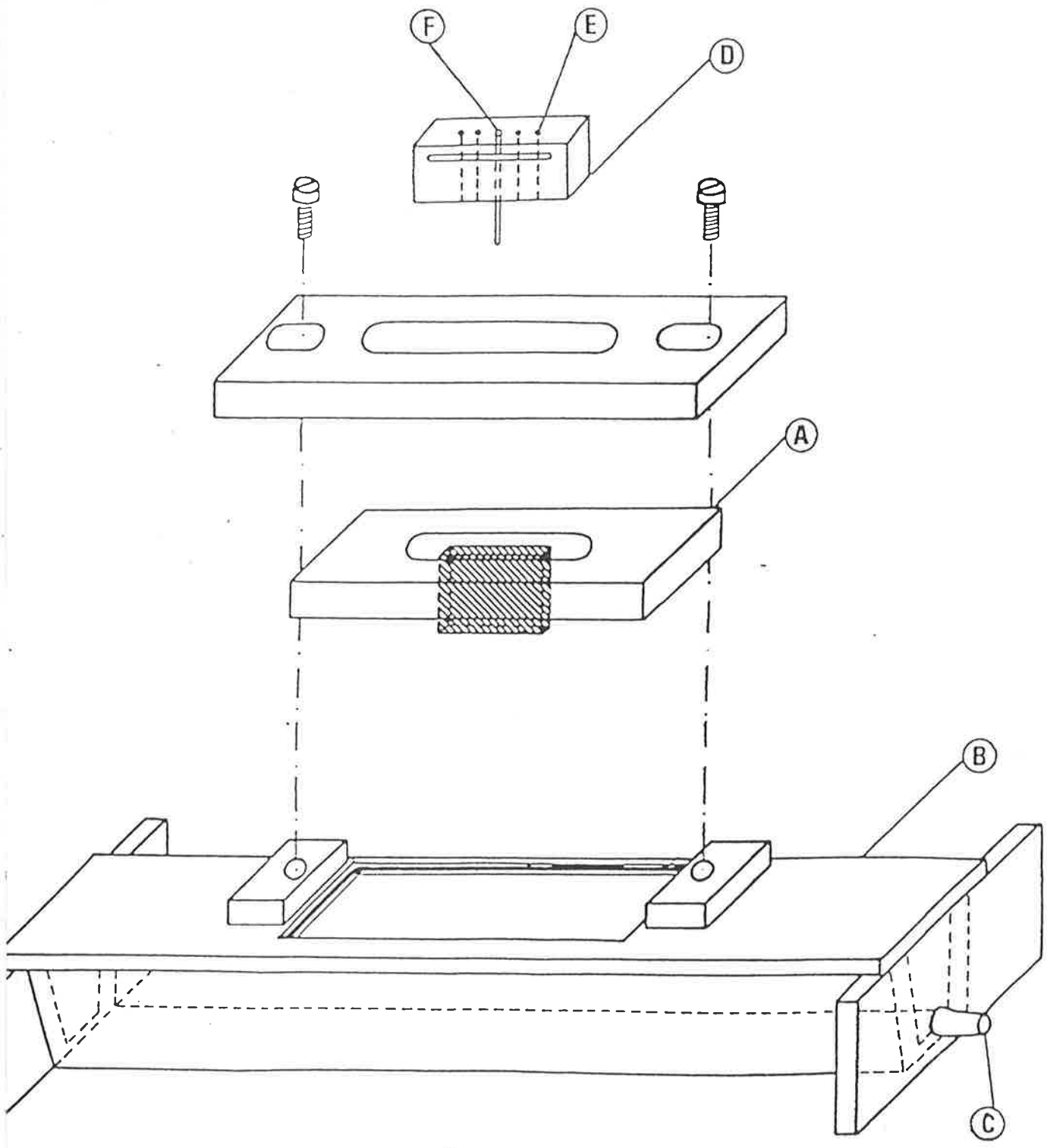
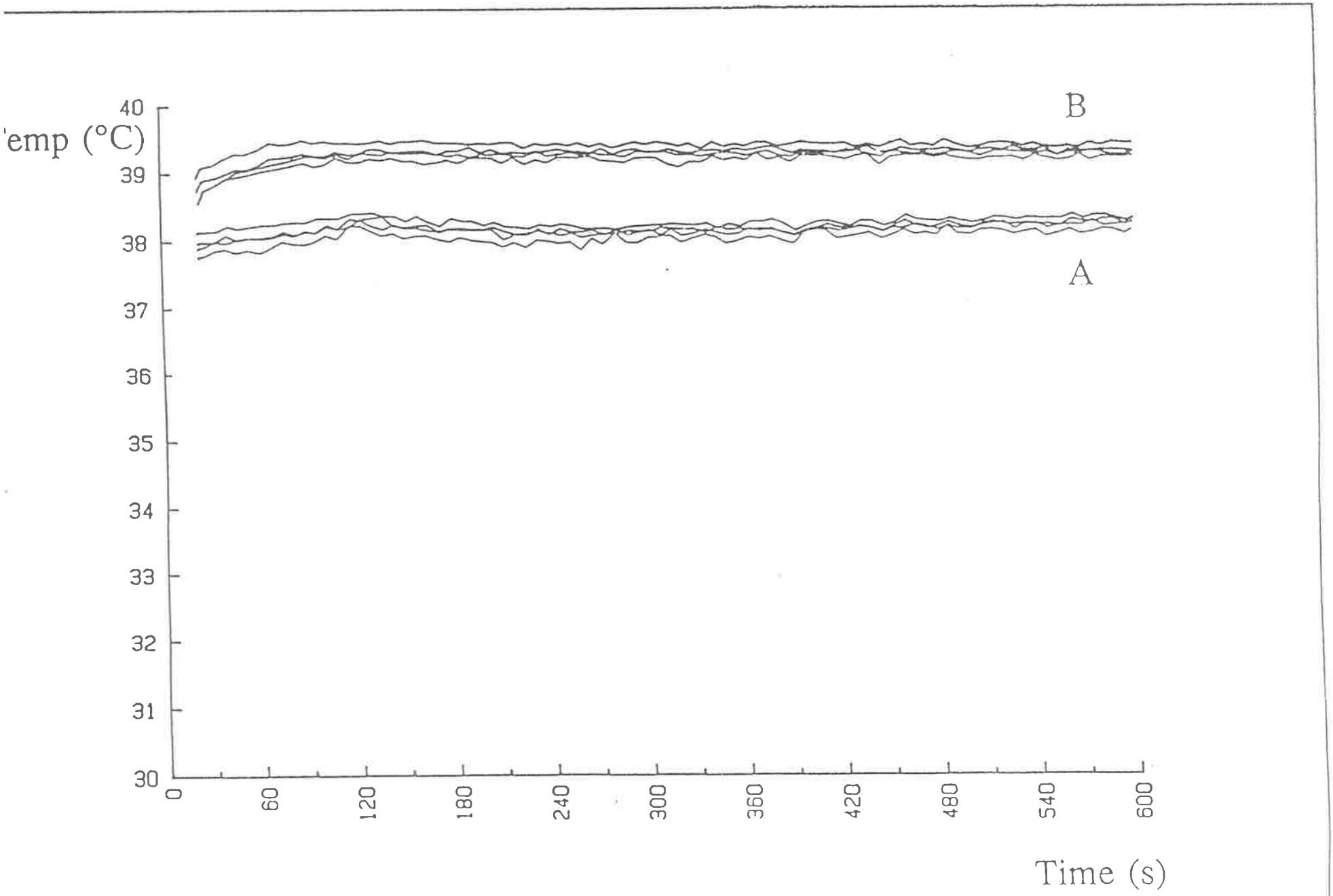


Fig. 1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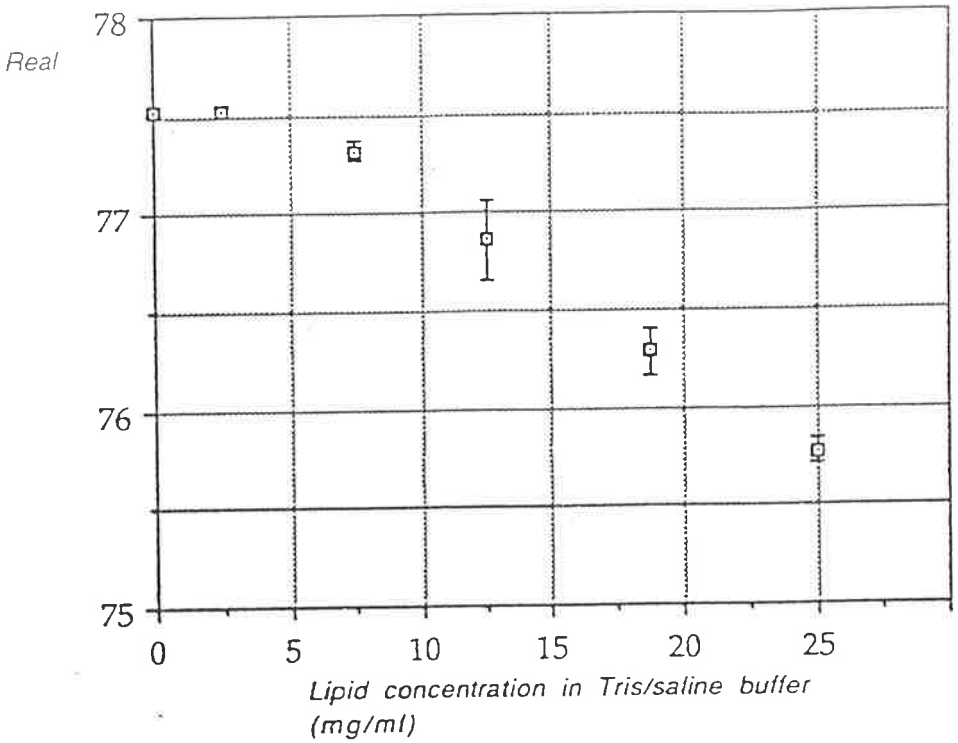
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Lipid concentration in Tris/saline buffer
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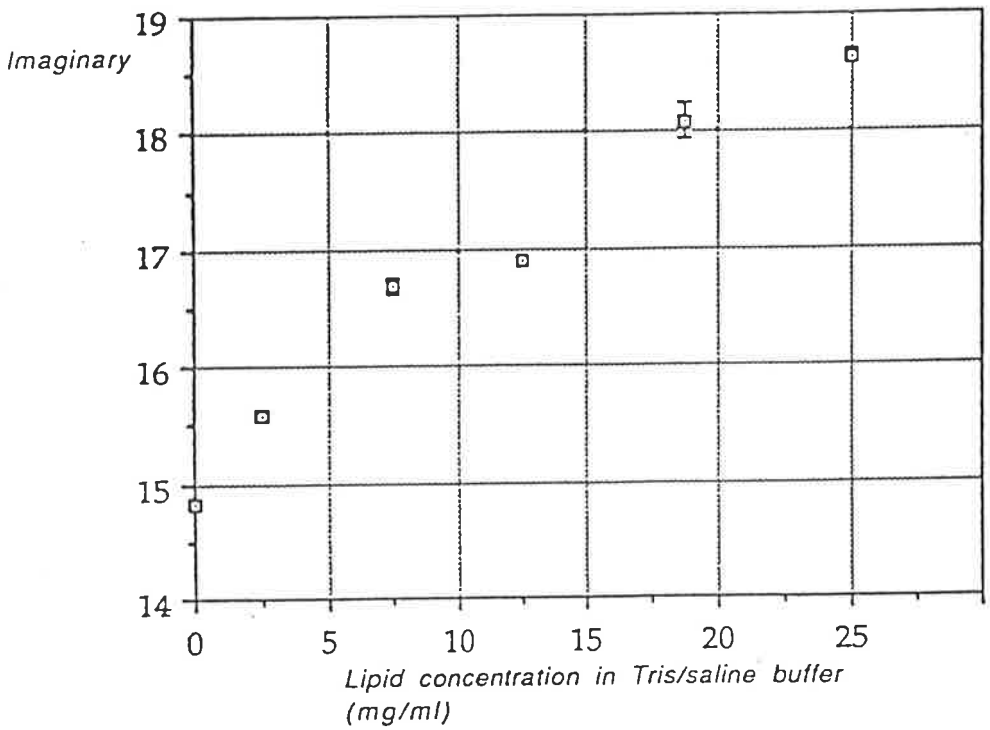
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Lipid concentration in Tris/saline buffer
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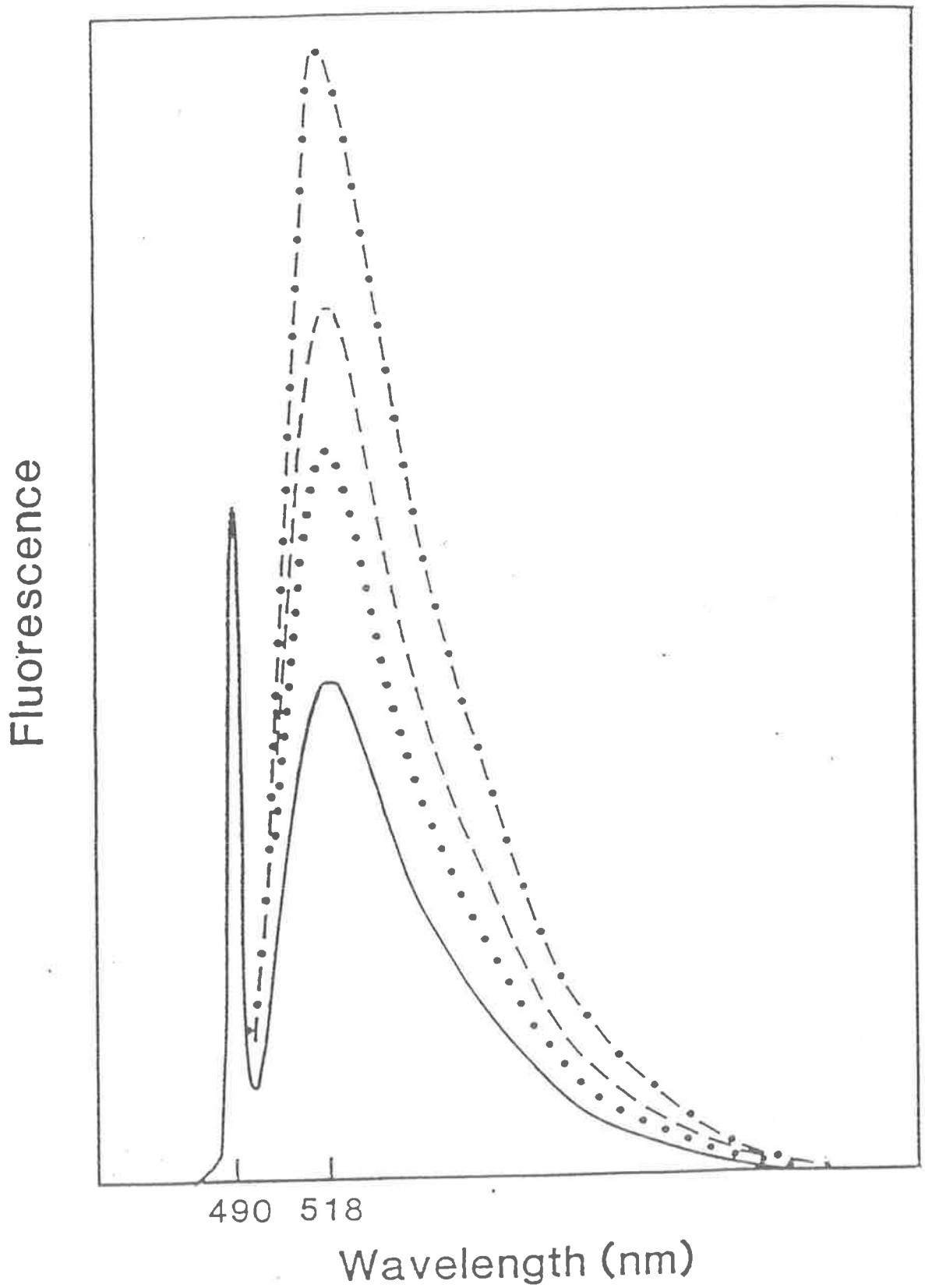

Fig 4