PHOSPHOLIPIDS AS ADJUNCTS FOR CHROMAFFIN GRANULE RELEASE

by

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( Department of Biochemistry )

We accept this thesis as confirming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
May 1981

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ABSTRACT

The chromaffin granules of adrenal medulla cells are membrane bound entities which act as storage vesicles for catecholamines, ATP, and protein. Release of contents to the extracellular medium appears to occur via calcium-stimulated exocytosis which involves fusion with the plasma membrane. The molecular mechanism of exocytosis was approached from two points of view.

First, it was shown by $^{31}$P-NMR techniques that the endogenous phospholipids assume a liquid-crystalline configuration at physiological temperature both in the intact membrane and in model systems composed of the extracted lipid. This is consistent with a structural role of phospholipids in vivo, serving to maintain membrane integrity. Addition of calcium to these systems resulted in little change in the biological membrane spectra and in the appearance of a relatively small component ($< 10\%$), possibly arising from phospholipid in the hexagonal H$_{II}$ phase in the model systems composed of the isolated total lipids.

Second, incubation of intact granules in the presence of upto 10 mM calcium did not cause significant release of contents. Similarly, incubation in the presence of exogenous lipid vesicles alone did not induce release. However, incubation with phospholipid systems, which have the ability to undergo structural transitions in the presence of calcium, followed by the introduction of calcium caused immediate and total release of granule contents. This behaviour is attributed to disruption
of membrane integrity arising from calcium induced fusion of the phospholipid vesicles with the granules. In contrast, incubation with phospholipid systems which do not undergo structural transitions in the presence of calcium is quite ineffective. On the basis of this information, a mechanism of calcium-induced exocytotic release of catecholamines in vivo is proposed.
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<tr>
<td>ATP</td>
<td>Adenosine-5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Diaminoethantetra-acetic acid</td>
</tr>
<tr>
<td>g</td>
<td>Centrifugal force</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-pipperazine ethanesulphonic acid</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide, oxidized</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide, reduced</td>
</tr>
<tr>
<td>³¹P-NMR</td>
<td>Phosphorus nuclear magnetic resonance</td>
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To Anuradha
Chapter I: INTRODUCTION

Membranes have been extensively studied over the past thirty years due to the awareness that many important biological processes in animal and plant cells and in microorganisms are mediated by membranes. This has led to various proposed models of membrane structure and also to the revelation of the enormous diversity of membrane-mediated functions, such as, intracellular compartmentalization of specific organelles, regulation of enzyme activities, facilitated transport, cell fusion, endo- and exocytosis, and cell-cell interactions. The purpose of this thesis is to gain some insight into the molecular mechanism of one of the above processes, namely exocytosis.

1.1 Definition of Membrane Fusion and Exocytosis

Membrane fusion is defined as the process by which two separate membranes join or unite to form a single membrane. This is illustrated in Figure 1, where two membrane bound vesicles fuse to form a single vesicle.

Figure 1. Membrane Fusion
It is obvious that prior to the fusion event, close apposition of the two membranes is necessary in order to allow some kind of interaction between the two fusing membranes which would promote fusion. After fusion, the contents of the two vesicles and their respective membranes become confluent.

Exocytosis is a specific membrane fusion event between the secretory vesicles of a cell and the cell plasma membrane. This fusion process results in the release of the vesicle products directly into the extracellular space. Exocytosis is basic to the processes of cell excretion and secretion and is involved in the release of a wide variety of enzymes, hormones, and neurotransmitter substances from such cells as the newly fertilized egg, blood platelets, leukocytes, mast cells, nerve cells, cells participating in the formation of kinins, angiotensin, and erythropoietin, and hormone-producing cells in the adrenal medulla, neurohypophysis, anterior pituitary, thyroid, and pancreas (reviews; 1, 2, 3). That the release of these secretory products is essential for homeostasis is self-evident. The localization of the secretory products in membrane-bound vesicles and their export from the cell by exocytosis offers several advantages. The products are protected against degradation from the cytoplasmic enzymes and can therefore be transported over fairly long distances, as in the case of nerve axons. The products can be released in quantal amounts in response to a direct physiological stimulus for release on the plasma membrane. For nerves, the stimulus is an electrical depolarization of the presynaptic terminal
(4) whereas for hormone secretion, the stimulus is usually a chemical which induces membrane depolarization (2), but in all the exocytotic processes investigated so far, calcium ions have been found to be vital for the operation of the secretion mechanism. Therefore, exocytosis is a specific membrane fusion event which is calcium dependent, however, the exact role of calcium is unclear.

Membrane fusion cannot be considered in isolation from the other aspects of membrane activity and analysis of the mechanism of membrane fusion must take into account the basic properties of the membranes.

1.2 Structure of Membranes

Membranes are composed predominantly of two major types of molecules: lipids and proteins. The lipids are thought to provide the fundamental building blocks of the membranes by forming a bilayer with their polar groups at the intracellular and extracellular surfaces and their hydrophobic fatty-acyl chains stacked roughly perpendicular to the plane of the membrane. This bilayer structure was first proposed theoretically by Gorter and Grendel (5) in 1925 and later confirmed through experimental evidence by Danielli and Davson (6) in 1935. It has since then been extensively supported by numerous independent techniques (e.g. X-ray diffraction (7), electron microscopy (8), freeze-fracture (9), and nuclear magnetic resonance (10)).

The bilayer model was extended by Singer and Nicolson in 1972 into a Fluid Mosaic model of biological membranes (11). The fluidity was
suggested to be due to the rotational and translational motion of the phospholipids arranged in a bilayer matrix, while the mosaic nature included integral proteins that penetrated into or through the membrane, and also peripheral proteins that were "attached" onto the membrane surface. The integral proteins have been shown to contain a higher percentage of non-polar amino acids which are presumably involved in hydrophobic interactions with the phospholipid fatty-acyl chains. The peripheral proteins are relatively polar and can easily be removed from the membranes by changes in ionic strength as they are largely attached by weak electrostatic bonds. The fluid mosaic model has been widely accepted due to its dynamic nature with respect to protein function. It predicts that the motion of proteins may be modified by the fluidity of the lipid bilayer.

1.3 Phase transitions and fluidity characteristics of lipids

A typical biological membrane contains over a hundred different species of lipids, predominantly phospholipids. A typical eukaryotic membrane, for example, may contain phosphatidylcholine (PC), sphingomyelin (Sph), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylinositol (PI), and cholesterol or other sterols. Each of the phospholipid species have associated with them a range of fatty acids of differing chain length and degree of saturation. Therefore, in order to unravel any functional role of these phospholipids, model systems using synthetic phospholipid of well defined
composition were developed.

1.3.1 Gel-Liquid Crystalline Phase Transition

The thermotropic behavior of a number of pure lipids was investigated by Chapman and coworkers (12) using the techniques of differential thermal analysis and differential scanning calorimetry. Pure phospholipids in aqueous dispersions were found to undergo a phase transition from a rigid gel-state to a fluid liquid-crystalline state. This transition temperature ($T_c$) is dependent upon the lipid class, acyl-chain length, degree of acyl-chain unsaturation, hydration, and ionic environment (13). In most well defined lipid systems, the transition is highly co-operative. Since a biological membrane consists of a large diversity of phospholipid species, the gel to liquid-crystalline transition is not so abrupt. Although gel-liquid crystalline transitions have been observed in pure lipid systems, they have been observed in a few biological membranes, such as in *Acholeplasma laidlawii*, relevance of this type of transition is still in doubt for *in vivo* situations.

1.3.2 Fluidity of Membranes

Fluidity of a biological membrane reflects the degree of order or disorder in the phospholipid acyl-chains. It can be measured by spectroscopic techniques, such as, electron spin resonance (14), nuclear magnetic resonance (15), or fluorescent polarization (16), and by differ-
ential scanning calorimetry techniques (13). The fluidity is related to the gel-liquid crystalline phase transition of the component individual lipids, that is, membranes containing a large percentage of unsaturated lipids will be more fluid than those containing a larger percentage of saturated lipids. Temperature, presence of cholesterol or protein in the bilayer also modify membrane fluidity.

Cholesterol is the predominant neutral lipid found in biological membranes and has dramatic effects on membrane fluidity. In a liquid-crystalline medium, cholesterol has a "condensation effect" by increasing the degree of order in the fatty-acyl chains and thereby decreasing fluidity. In a gel-state medium, it has a "liquifying effect" by preventing crystallization of the phospholipids (18). Therefore, the presence of cholesterol in a biological membrane has been implicated in maintaining the fluidity of the bilayer.

The biological significance of fluidity in a biomembrane is as yet unclear. It has been suggested to be of importance to the permeability of membranes, for example, in trans-membrane ion fluxes, or in controlling enzyme activity. Evidence for the former has resulted from permeability studies correlating the effect of fatty-acyl chain composition of phosphatidylcholine membranes to the permeability to glycerol or erythritol (19), and evidence for the latter has resulted from observations of discontinuities in Arrhenius plots of membrane-bound enzyme activities which have been attributed to the "melting" of the annular
lipid shell around the enzyme (20).

1.3.3 Polymorphic Phase Transition

Apart from the gel-liquid crystalline phase transition, phospholipids also experience a polymorphic phase transition from the liquid-crystalline bilayer structure to a hexagonal $H_{II}$ configuration or another non-bilayer structure (21). This polymorphic phase transition occurs within 10°C of the gel-liquid crystalline transition and is also sensitive to temperature, lipid composition, pH, and divalent cations (22). Studies on isolated pure phospholipid species have revealed that a significant proportion of the phospholipids in a biological membrane would preferentially adopt in isolation a hexagonal $H_{II}$ configuration at physiological temperatures. Important examples of such lipids include unsaturated phosphatidylethanolamines (21,22,23), monoglcusyldiglyceride (24), as well as phosphatidic acid (25) and cardiolipin (25) in the presence of calcium. In addition, lipids such as cholesterol (21,22,27) and unsaturated fatty acid (28) have been shown to induce the formation of hexagonal $H_{II}$ phases from bilayer systems. The effect of pH in inducing the hexagonal $H_{II}$ has also been demonstrated in the case of phosphatidylserine, where the $H_{II}$ phase was induced by lowering the pH to 3.0 (29).

The polymorphic behaviour of phospholipids and their possible functional roles in biological membranes has been recently reviewed by Cullis and De Kruijff (30). They have suggested non-bilayer structures
such as the hexagonal \( \text{H}_{\text{II}} \) and inverted lipidic micellar structures occur as possible intermediates in membrane-mediated processes involving membrane fusion and transbilayer transport of lipids (flip-flop).

1.3.4 Asymmetric nature of biological membranes

Most of the membranes studied so far have been shown to have asymmetry with respect to their lipid, carbohydrate, and protein environment in the two bilayer leaflets. The best documented evidence comes from the erythrocyte cell membrane. Protein labelling studies, phospholipid modification and phospholipase treatment studies, and lectin binding experiments (31) have indicated that the outer leaflet is composed of oligosaccharides containing terminal glucose or mannose sugar residues, contains two major proteins (acetylcholinesterase and 5'-nucleotidase), and is predominantly composed of phosphatidylcholine and sphingomyelin phospholipids. The inner cytoplasmic leaflet is associated with most of the proteins (spectrin, myosin and actin-like proteins, and protein kinase) and is predominantly composed of aminophospholipids such as phosphatidylethanolamine and phosphatidylserine (32).

The presence of asymmetry can be rationalized by the fact that the reactions occurring on the inner and outer leaflets of a biological membrane are quite distinct and therefore would require different environments. There is now good evidence for the specific role of carbohydrates in processes involving cell adhesion and recognition (33),
and the activities of the proteins on the inner leaflet has been associated with various cellular reactions. However, the reason whereby phospholipid asymmetry is maintained is unknown.

As mentioned earlier, membrane fusion occurs ubiquitously in nature. The fusion event has been shown to involve the interactions between the two phospholipid monolayers of the opposing membranes. For example, in exocytosis, the fusion event involves direct interaction between the outer monolayer of the secretory vesicle and the inner monolayer of the plasma membrane. This has been implied from electron microscopic studies (34). Furthermore, freeze-fracture studies of the exocytotic event in chromaffin cells (35) and mast cells (36) have demonstrated that the membrane proteins segregate away from the fusion site and are therefore probably not directly involved during the fusion reaction. The proteins might possibly be involved in bringing about close apposition of the two membranes and in the recognition of the fusion site on the plasma membrane, but the fusion event itself clearly would involve some sort of structural transition of the phospholipids. Hence, phase transitions, fluidity, and asymmetric distribution of phospholipids could be important factors in allowing fusion to occur.

This thesis investigates the possible role of phospholipid specificity in the process of membrane fusion. The fusion system studied is the chromaffin granule which specifically fuses with the chromaffin cell plasma membrane by the process of exocytosis. The ease of isolation,
low cost, and the extensive characterization of the chromaffin granules make it a convenient system for studying membrane fusion.

1.4 The Chromaffin Granule: Structure and Function

1.4.1 Historical Perspective

The first indication that the hormones of the adrenal medulla, noradrenaline and adrenaline, were stored in a subcellular particle was obtained by Blashko and Welch (37) and by Hillarp and Hokfelt (38) in 1953. They showed that the bulk of the catecholamines resided in the fraction obtained by high speed centrifugation after removing the cell debris by a preceding low speed centrifugation step. Electron micrographs of the adrenal medulla by Lever (39) in 1955 showed membrane bound vesicles (200nm dia.) which were smaller than the mitochondria and he suggested that these stored the hormones of the adrenal medulla. Sjostrand and Wetzstein (40) in 1956 obtained similar micrographs and introduced the term, chromaffin granules. Morphological and biochemical characterization was subsequently correlated by Hagen and Barrnett (41) and it was concluded that the chromaffin granules were distinct organelles which were the site of storage of the catecholamines in the adrenal medulla.

1.4.2 Composition of the Chromaffin Granule

The two components of the chromaffin granule, water-soluble (i.e.
granule contents) and water-insoluble (i.e. granule membrane) fractions can easily be separated by lysis of the chromaffin granules in a hypo-osmotic buffer solution. The composition of the contents and of the membrane are listed in Table I. (42)

Table I: Composition of the bovine adrenal chromaffin granule

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount, % total dry wt</th>
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<tbody>
<tr>
<td>soluble content</td>
<td></td>
</tr>
<tr>
<td>catecholamines</td>
<td>20.5</td>
</tr>
<tr>
<td>adenine nucleotides</td>
<td>15</td>
</tr>
<tr>
<td>protein</td>
<td>27</td>
</tr>
<tr>
<td>calcium</td>
<td>0.1</td>
</tr>
<tr>
<td>magnesium</td>
<td>0.02</td>
</tr>
<tr>
<td>membrane</td>
<td></td>
</tr>
<tr>
<td>phospholipid</td>
<td>17</td>
</tr>
<tr>
<td>cholesterol</td>
<td>5</td>
</tr>
<tr>
<td>protein</td>
<td>8</td>
</tr>
<tr>
<td>calcium</td>
<td>0.06</td>
</tr>
<tr>
<td>magnesium</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The soluble contents are rich in catecholamines (0.71 M), adenine nucleotides (0.13 M ATP), and proteins (210 mg/ml). The soluble proteins are termed chromagranins. There are at least twelve different chromagranins present but their functions are still unknown. The only protein with known function is dopamine-\(\beta\)-hydroxylase, which is predominantly found in the membrane.

The water-insoluble proteins (presumably membrane proteins) are termed chromembrins and include dopamine-\(\beta\)-hydroxylase, Mg\(^{2+}\)-activated ATPase, NADH oxidoreductase, phosphatidylinositol kinase, and cytochrome b\(^{559}\). Spectroscopic analysis have also found a flavoprotein. Charact-
erization and topography of the glycoproteins by Winkler et al. (43) showed that the carbohydrate portions of the glycoproteins faced the luminal side of the granule. Recently, Abbs and Phillips (44) investigated the organization of the chromamembrins and found that most of the proteins were accessible on the cytoplasmic side of the granules with at least two proteins spanning the membrane.

The chromaffin granules are relatively rich in lipids with a protein to lipid ratio of 0.45 (w/w) in the membrane. The lipids are chiefly cholesterol and phospholipids with a characteristic relatively high concentration of lysolecithin (about 15%). Table II lists the average phospholipid composition of the chromaffin granule membrane.

Table II: Phospholipid composition of the Chromaffin Granule Membrane.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Percentage Composition*</th>
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<tr>
<td>Phosphatidylcholine</td>
<td>27</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>34</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>9</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>9</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>13</td>
</tr>
<tr>
<td>Lysophosphatidylcholine</td>
<td>15</td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td>1</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td></td>
</tr>
</tbody>
</table>

*Average of all the literature values including the author's (45).

So far, it has been difficult to elucidate the phospholipid asymmetry of the membrane because about 50% of the total phospholipids have been found to be resistant to phospholipases (45,46). Although the
nature of this protection is unknown, phospholipase degradation of all the phospholipids after heat-treatment of the granules suggests that it could be due to binding with the membrane proteins (45).

1.4.2 Dynamic Role of the Chromaffin Granule

The chromaffin granule does not function as an inert reserve of catecholamines in the adrenal medulla, but also stores several other secretory products and is directly involved in the biosynthesis and secretion of catecholamines. Figure 2 illustrates some of the dynamic aspects of the chromaffin granule inside the chromaffin cell.

Storage

Apart from the significant store of catecholamines (0.71 M), the contents also include large quantities of adenine nucleotides (0.13 M ATP) and proteins (210 mg/ml) (47). The total amount of stored substances account for about 63% of the dry weight of the chromaffin granule. The high concentration of ATP has been suggested to be involved in maintaining high concentrations of catecholamine by formation of high molecular weight complexes (48).

Synthesis

The chromaffin granule contains the enzyme dopamine-β-hydroxylase which plays a vital role in the biosynthesis of catecholamines. Since the cytosol of the chromaffin cell has potent inhibitors of this enzyme, the conversion of dopamine to noradrenalin occurs inside the chromaffin
granule. This has been supported by $^3$H-tyrosine pulse-labelling experiments and also by the finding that reserpine, which inhibits active uptake of dopamine into the chromaffin granule, inhibits synthesis of noradrenalin in slices of adrenal medulla (49).

**Secretion**

The ultimate role of the chromaffin granule is in the release of the secretory products into the blood, thus fulfilling the physiological function of the adrenal medulla. The release is accomplished by exocytosis which involves specific fusion of the granule membrane with the chromaffin cell plasma membrane. Jacobj (50) in 1892 demonstrated that electrical stimulation of the splanchnic nerve or direct stimulation of the adrenal gland resulted in secretion of biologically active substances. Feldberg and coworkers (51) later showed that acetylcholine was the physiological stimulus for secretion and found both nicotinic and muscarinic receptors on the chromaffin cells. Finally, extensive studies by Douglas and coworkers (52) found calcium to be the universal and necessary step in this stimulus-secretion coupling event. The molecular mechanism of release has yet to be elucidated.
Figure 2. Subcellular dynamics of the chromaffin cell. Release of chromaffin granule (CG) contents occurs by exocytosis. The empty membrane may pinch off to give rise to several small coated vesicles (CV). The CV lose their coating and are either degraded by lysosomal enzymes in the multivesicular bodies (MVB) or may regenerate back into a chromaffin granule. CP, coated pit; Mito, mitochondria.
1.5 **Approach to the problem of elucidating the molecular mechanism of exocytosis.**

The objective of this thesis was to gain some insight into the molecular mechanism of exocytosis. Since this membrane fusion event involves direct interaction between the phospholipids of the chromaffin granule membrane and the plasma membrane, the problem was approached from two points of view. First, the dynamic organization of the chromaffin granule membrane and isolated lipids was investigated by phosphorus nuclear magnetic resonance ($^{31}$P-NMR) techniques. This was to ascertain if the specialized chromaffin granule membrane was different from other biological membranes and whether any differences observed could be correlated to functions such as fusion. In particular, it was important to characterize the bilayer and non-bilayer preferences of the endogenous lipid and the influence of calcium, given recent suggestions that non-bilayer lipid configurations occur as intermediates in fusion of model (53) and biological (28) membrane systems, and that calcium can trigger formation of such structures in certain lipid systems (26,54). Secondly, from the point of view of efficient extracellular release of catecholamines, it is obviously advantageous if granule–plasma membrane as opposed to granule-granule fusion is preferentially stimulated by the presence of calcium. Therefore, the influence of specific exogenous lipid model membranes, which may approximate the inner monolayer of the chromaffin cell plasma membrane or prefer either bilayer or non-bilayer configurations in the presence of calcium, on
calcium-stimulated release of chromaffin granule contents was investigated. Hence, specificity of phospholipids in promoting release of chromaffin granule contents was determined.

1.6 Use of $^{31}$P-NMR in determining membrane structure

Since biological membrane lipids are predominantly phospholipids, $^{31}$P-NMR is an attractive non-perturbing tool to investigate the motion and average orientation of the phosphate groups. The detection of phospholipid polymorphism by $^{31}$P-NMR techniques rests on three factors (30).

First, a large chemical anisotropy is exhibited by the phosphorus nuclei, which for large liquid-crystalline bilayer systems (>2000 Å) is only partially averaged by the restricted modes of motion available. These consist primarily of rapid rotation of the molecules along their long axis. Under proton decoupling conditions, it is possible to remove the P-H dipolar interactions and this results in a characteristic broad spectrum with a low field shoulder and a high field peak separated by approximately 40 ppm. for large lamellar organizations. A typical bilayer spectrum is illustrated in figure 3a.

Second, all glycerol-based phospholipids (PC, PE, PS, PG, PI), except PA, and sphingomyelin have a similar lineshape when in the liquid-crystalline bilayer. Therefore, in a mixed lipid system, all the endogenous phospholipids that are in a bilayer contribute to a composite lineshape.
Finally, in a bilayer system the third factor contributing to the $^{31}$P-NMR spectrum is the lateral diffusion of the phospholipids. In large bilayer structures, such as liposomes and biological membranes, the process of lateral diffusion is not fast enough on the NMR time-scale ($10^{-5}$ seconds) to produce an effective motional averaging mechanism. In contrast, in small sonicated vesicles or other small structures, the ability of phospholipids to diffuse laterally around the vesicle and vesicle tumbling produce line-narrowing effects. These isotropic motional averaging effects are observed for lipids in inverted micellar configurations and in other small structures, such as the cubic or rhombic phases. A narrow isotropic signal is therefore difficult to interpret by $^{31}$P-NMR techniques alone.

The other non-bilayer structure of interest is the hexagonal $H_{II}$ phase. As shown in figure 3b, the hexagonal $H_{II}$ configuration consists of long cylinders of phospholipids whose polar headgroups are oriented towards small (20 Å diameter) aqueous channels. Such structures experience additional motional averaging as compared to the large bilayer structures due to lateral diffusion around the aqueous channels. Hence, the hexagonal $H_{II}$ phase exhibits a characteristic $^{31}$P-NMR lineshape which has the reverse asymmetry compared to the bilayer lineshape and is narrower by a factor of two. A summary of all three lineshapes are illustrated in figure 3.

Close correlation between the polymorphic phase behaviour of phospholipids as detected by $^{31}$P-NMR, freeze-fracture, and X-ray studies (26)
confirms the validity of the $^{31}$P-NMR technique for determining membrane structure.


Corresponding $^{31}$P NMR spectra of phospholipid phases.

- Bilayer
- Hexagonal (H$_1$)
- Phases where isotropic motion occurs:
  - a. Cubic
  - b. Rhombic
  - c. Micellar, inverted micellar
  - d. Vesicles

Figure 3. $^{31}$P-NMR spectra of phospholipid phases.
2.1 Isolation of Chromaffin Granules

A modification of the methods of Smith and Winkler (55) and of Helle et al. (56) was used to isolate the chromaffin granules from bovine adrenal medulla. Between twenty and thirty fresh bovine adrenal glands were obtained from a local abattoir, where they were placed on ice until ready for isolation. All subsequent steps were carried out at 4°C.

2.1.1 Preparation of the Large Granule Fraction

The adrenal glands were defatted and dissected free of cortical tissue. The adrenal medullae were placed in ice-cold 0.3 M sucrose solution containing 10 mM HEPES and 1 mM EDTA at pH 7.0 (this solution is referred to as "buffered sucrose"). Homogenization of the adrenal medullae was more conveniently carried out employing 40-100 mesh sand prepared by the method of Guena (57). The homogenate was filtered through glass wool and centrifuged at 755 g for 10 min. The pellet (cell debris) was discarded and the low speed supernatant was centrifuged at 17,000 g for 10 min. The upper fluffy brown layer on the pellet (mitochondria and lysosomes mainly) was carefully decanted and the pink pellet was washed with buffered sucrose. The high speed centrifuge step was repeated twice after resuspending the pink pellet containing the chromaffin granules in 40 ml of buffered sucrose. The final
pellets obtained after the high speed centrifugations were pooled and resuspended in approximately 10 ml. of buffered sucrose to give a protein concentration of around 30 mg/ml. This fraction contained partially purified chromaffin granules and was referred to as the "Large Granule Fraction" (LGF).

The large granule fraction was stable in an isoosmotic medium and had a small amount of mitochondrial contamination (see purity section 2.3). In the majority of the release experiments, the large granule fraction rather than the highly purified granules was employed because of the osmotic fragility of the pure preparations.

2.1.2 Preparation of the highly purified chromaffin granules

Due to the high density of the chromaffin granules relative to that of the mitochondria and lysosomes, the chromaffin granules can be highly purified by centrifugation through a hyperosmotic sucrose medium.

About 2 ml of the large granule fraction was layered onto 30 ml. of 1.6 M sucrose and centrifuged at 80,790 g for 1 hr. Several different layers resulted after the step-gradient centrifugation. These are illustrated and numbered in figure 4. Analysis of the fractions by Smith and Winkler (55) have shown that the interface layers 2 and 2' contain most of the mitochondrial and lysosomal activity. The pink sediment, 5, corresponded to the highly purified chromaffin granules.
Summary of the isolation procedure for chromaffin granules of bovine adrenal medulla.

Step (4) was repeated twice.
and were used for lipid extraction and determination of the phospho-
lipid composition of the chromaffin granule membrane. The scheme of
isolation is illustrated in figure 4.

2.2 Isolation of the chromaffin granule membrane

The chromaffin granule membranes were prepared by lysis of the
chromaffin granules in a hypoosmotic medium (5 mM HEPES, pH 7.0), follow-
ed by a freeze-thaw cycle, and subsequent centrifugation at 27,000 g
for 30 min. This was repeated four or five times until the absorbance
of the supernatant at 265 nm after centrifugation was less than 5 %
of the initial value. The brown pellet obtained after the centrifugation
step corresponded to the chromaffin granule membrane fraction.

2.3 Determination of mitochondrial contamination in the
large granule fraction

Since the major contaminant of the chromaffin granule preparation
was mitochondria, it was of interest to determine the extent of impurity
in the large granule fraction. The enzyme, malate dehydrogenase (MDH),
was used as a mitochondrial marker enzyme. MDH catalyzes the follow-
ing reaction in the mitochondrial matrix:

\[
\text{Oxaloacetate} + \text{NADH} + \text{H}^+ \rightarrow \text{L-Malate} + \text{NAD}^+
\]

NADH absorbs at 340 nm, therefore the decrease in absorbance over time
was taken as corresponding to MDH activity. The \( A_{340}/\text{min/mg protein} \)
was taken as the specific activity of MDH, which was proportional to
the amount of mitochondria in the sample. Fraction 2 in the ultracentrifugation step of the preparation was assumed to correspond to the pure mitochondrial fraction.

The stock solutions needed for the assay were 0.1 M phosphate buffer, pH 7.5, 15 mM oxaloacetate solution (2 mg/ml. in phosphate buffer), and 12 mM NADH (10 mg/ml). The reaction was preformed in a 5 ml cuvette by adding the ingredients in the following order: 2.83 ml phosphate buffer, 0.10 ml oxaloacetate solution, 0.05 ml NADH solution, giving a final concentration of 95 mM, 0.5 mM, and 0.2 mM respectively. The reaction was started by addition of 0.02 ml. of the sample. The decrease in absorbance was recorded for about a minute at 340 nm. The reference cell had all the ingredients except the NADH solution.

The specific activity of MDH in the large granule fraction and the highly purified chromaffin granules was approximately 15 % and 5 % of the pure mitochondrial fraction respectively.

2.4 Lowry Protein Assay

Protein was measured according to the procedure of Lowry et al. (58), using crystalline bovine serum albumin as a standard. Stock solutions required for the assay were 2 % Potassium Tartate, 1 % Copper Sulfate, and 2 % Sodium Carbonate dissolved in 0.1 N Sodium Hydroxide. Solution I was freshly prepared by mixing 98.0 ml of Sodium Carbonate with 1.0 ml. of Potassium Tartate and 1.0 ml. of Copper Sulfate. Solution
Figure 5. Protein standard curve
II was prepared by diluting 2 N Folin's Reagent to 1 N.

The assay was carried out by adding 5.0 ml. of solution I to 1.0 ml of sample and water. After 10 min, 0.5 ml. of solution II was added followed by immediate vortexing. Absorbance at 550 nm was recorded after 30 min. A typical Lowry standard curve is shown in figure 5.

2.5 Lipid Isolation and Purification

2.5.1 Lipid Extraction from Chromaffin Granule Membranes

Lipids were extracted by the procedure of Bligh and Dyer (59). Briefly, a sample of chromaffin granule membranes was diluted to 5 ml with water. Another 2.1 volumes of methanol and 1.0 volume of chloroform was added and the one-phase solution was stirred for 15 min. To extract the lipids, the solution was made into two-phases by the addition of 1.0 volume of chloroform and 1.0 volume of water. The cloudy solution was centrifuged on a bench-top centrifuge at approximately 3,000 rpm for 5 min. The top water-soluble fraction (containing proteins, ions, etc.) was aspirated off and the remaining chloroform phase containing the lipids was flash-evaporated in a round bottom flask under vacuum. The dry lipid was redissolved in some chloroform and stored at -20°C under nitrogen.

2.5.2 Isolation of erythrocyte membrane phospholipids

The erythrocyte membrane phospholipids were a gift from Dr. M.J.
Hope. They had been isolated from human erythrocytes and purified using low pressure liquid chromatography on silicic acid and carboxymethyl cellulose columns. The lipids were eluted by mixtures of chloroform and methanol, and were > 99% pure with respect to phosphorus (60).

Chloroform mixtures of the outer erythrocyte monolayer consisted of 44 mol % PC, 44 mol % Sph, and 12 mol % PE, with an equimolar amount of cholesterol. The inner monolayer mixture consisted of 47 mol % PE, 28 mol % PS, 15 mol % PC, and 10 mol % Sph, with an equimolar amount of cholesterol (61).

2.5.3 Isolation and purification of Phosphatidylcholine from egg yolk and soya beans.

Crude soya PC was purchased from Sigma while crude egg PC was isolated from egg yolk as follows. Thirty egg yolks were stirred intensively in 1.25 L of acetone in order to precipitate the lipids which were subsequently filtered through a course G3 glass filter. The precipitate was washed with 2 L of acetone and the lipids were extracted three times with 500, 400, and 300 ml of chloroform/methanol (1:1) by stirring for 5 min and subsequent filtration. The combined filtrate was flash-evaporated under vacuum and residual lipids were dissolved in about 100 ml of chloroform.

To obtain a partially purified preparation of PC, the crude lipid solution (about 100g) was purified by Aluminum Oxide chromatography. One kilogram of Aluminum Oxide (Al₂O₃) was suspended in chloroform/
methanol (1:1) and filtered over a coarse glass filter in order to remove most of the fines. The washed Al$_2$O$_3$ was then suspended in chloroform and packed into a 200 x 5 cm glass column. The column was washed with 1.5 L of chloroform and the crude PC lipid solution was loaded onto the column. Neutral lipids (triglycerides and cholesterol) were eluted with 1.0 L of 95 % chloroform-methanol at a flow rate of 6 ml/min and the partially purified PC fractions were quickly collected by washing the column with 50 % chloroform-methanol at maximum flow rate in order to prevent PC degradation to lysoPC on the column. All fractions containing PC and no lysoPC were pooled and dried down by flash-evaporation.

The partially purified PC fraction was then highly purified by High Pressure Liquid Chromatography (HPLC) on the Waters Prep LC-500 liquid chromatography system as described by Patel and Sparrow (62). Briefly, the compressed silica gel column was washed with 2.2 L of chloroform-methanol-water (60/40/10) and re-equilibrated with chloroform-methanol-water (60/30/4). About twenty grams of partially purified PC dissolved in chloroform (1 gm/ml) was applied to the column and eluted at a flow-rate of 100 ml/min. One hundred milliliter fractions were collected and the phospholipid elution profile was followed by thin layer chromatography on glass slides. Fractions containing absolutely pure PC were combined and dried down by flash-evaporation arriving at white compounds which were >99 % pure PC.
The pure soya PC was used to synthesize its respective PE and PS by employing the base-exchange capacity of phospholipase D. Egg PS was also synthesized similarly while egg PE was purified from total egg yolk lipids by HPLC.

2.5.4 Preparation of Phospholipase D

Phospholipase D, which hydrolyzes the headgroups of phospholipids, is very useful for the synthesis of less abundant phospholipids, such as phosphatidylserine. A study of phospholipase D activity in various plant tissues by Davidson and Long (63) found that Savoy cabbages was the richest source of the enzyme. Hence, a partially purified preparation of phospholipase D from fresh Savoy cabbages according to their procedures was utilized to synthesize PS and PE.

Phospholipase D purification was done as follows. The inner light-green leaves of Savoy cabbages (4 Kg) were homogenized in a Waring blender at maximum speed in 3 L. of ice-cold water for 3 min. intervals. The homogenate was freed from fiber by squeezing through four layers of cheesecloth and then centrifuged at 13,000 g. for 30 min. The pH of the supernatant was adjusted to 5.5 with 4 N HCl and 250 ml fractions were quickly heated to 55°C in a boiling water-bath and then immediately cooled to 0°C. The heat-treated filtrate was spun again at 13,000 g. for 30 min and added to 2 volumes of ice-cold acetone with continual stirring in order to precipitate the proteins. The acetone precipi-
pitate was allowed to stand for 2 hr. Subsequently, most of the yellowish supernatant was aspirated off and the precipitate was transferred into metal GSA tubes and spun at 1,000 g for 5 min. The white pellet was lyophilized in order to remove all the residual acetone in the preparation. The dry powder was resuspended in 75 ml of 0.2 M sodium acetate buffer, pH 5.6, containing 0.04 M calcium chloride and continually stirred for 30 min in an ice-cold water bath. The solution was then transferred to an SS-34 centrifuge tube and spun at 17,000 g for 10 min. The brown supernatant, corresponding to the partially purified phospholipase D fraction, was carefully decanted into six test-tubes and stored at -20°C until used.

2.5.5 Preparation of Phosphatidylserine by Base-Exchange reaction

Both egg and soya PS were synthesized from their respective PC by employing the base-exchange capacity of phospholipase D according to the procedures of Comfurius and Zwaal (64). The transphosphatidylation reaction catalyzed by phospholipase D was carried out in the presence of excess L-serine either with egg PC or soya PC.

The phosphatidylcholines were dissolved in anhydrous ethyl-ether at a concentration of 20 mg/ml. L-serine was first lyophilized to remove traces of methanol and subsequently dissolved at 45°C up to saturation (46% w/w) in 100 mM Acetate buffer (pH 5.6) containing 100 mM CaCl₂. One tube of partially purified phospholipase D was added
to the serine solution and an equal volume of the phosphatidylcholine solution in ether was also added. The incubation flask was immediately closed and shaken continuously in a water-bath at 35°C. Incubation was stopped after 35 min and pressure in the nalgene incubation flask was relieved by cooling it under a stream of cold water. The mixture was transferred to a glass centrifuge bottle and spun at 1,000 g for 10 min. The top ether phase was carefully transferred into a round bottom flask and the phospholipids were extracted once more from the incubation mixture with about 50 ml of ether. The combined ether fractions were flash-evaporated and the phospholipid was washed as follows in order to remove any water soluble components. The phospholipid was dissolved in 25 ml of chloroform-methanol (2:1), followed by addition of 6 ml of water. The two phases were mixed with a pasteur pipette and then centrifuged at 1,000 g for 5 min. The top aqueous layer, containing the water-soluble components was aspirated off and the chloroform lipid phase was dried down under nitrogen. Analysis of the total phospholipids showed that at least 20-30 % of the phosphatidylcholine had been converted to phosphatidylserine.

Phosphatidylserine was purified from the reaction mixture by carboxymethyl-cellulose (CM-cellulose) chromatography as described by Comifurius and Zwaal (64), except that the elution of the phospholipids was done by a continuous chloroform-methanol gradient rather than by a step-gradient. The pure PS fractions were pooled together and converted
to the sodium salt by dissolving the dry lipid in an acidic Bligh and Dyer monophase (chloroform/methanol/0.4 M HCl) which was subsequently titrated to pH 7.5 with a Bligh and Dyer monophase where the aqueous component was 0.5 M NaCl and 1.0 M NaOH. Addition of 0.4 volumes of water and chloroform to the total titrated volume resulted in a two-phase system. The chloroform phase containing the sodium salt of phosphatidylserine was dried down under nitrogen. The phosphatidylserine was shown to be > 99 % pure by two-dimensional thin layer chromatography.

2.5.6 Preparation of Soya Phosphatidylethanolamine

Soya phosphatidylethanolamine was also prepared by the base-exchange capacity of phospholipase D. The procedure was identical to the one described for the preparation of phosphatidylserine except that the reaction mixture contained 15 % (w/v) of ethanolamine instead of L-serine. The ethanolamine was neutralized prior to incubation by the addition of concentrated HCl. The transphosphatidylation reaction in this case was much more complete with approximately 80 % of the phosphatidylcholine being converted to phosphatidylethanolamine. The purification of the phosphatidylethanolamine was done by HPLC as described below.

2.5.7 Purification of Egg and Soya Phosphatidylethanolamine

Both egg and soya phosphatidylethanolamines were purified by HPLC
techniques as described by Patel and Sparrow (62). Egg PE was purified from a partially pure fraction obtained during the purification of egg PC by HPLC, while the soya PE was purified from the products of the transphosphatidylation reaction catalyzed by phospholipase D as described in section 2.5.6.

The compressed silica gel column of the HPLC was washed with 2.2 L of chloroform-methanol-water (60/40/10) and 1.0 L of chloroform-methanol-water (60/30/2). The column was then equilibrated with 2.0 L of chloroform-methanol-water (60/30/2). Between 5-10 gm of partially purified PE dissolved in chloroform at a concentration of 1 gm/ml. was applied to the column and the phospholipids were eluted at a flow-rate of 100 ml/min into 150 ml fractions. The pure PE fractions were pooled and flash-evaporated arriving at white compounds which were > 99 % pure. Apart from the TLC analysis of PE, the bilayer to hexagonal \( H_{II} \) transition temperature is very critical to the purity of PE. For example, the transition temperature of soya PE is 15°C and that of egg PE is 25°C, and an impurity of upto 1 % can shift this transition temperature of PE by 10°C. Therefore, determination of the characteristic transition temperature of PE was also essential in accessing the purity of egg or soya PE. The procedure is described in section 2.9.1

2.6 Thin Layer Chromatography (TLC)

TLC is considered to be the most effective and versatile technique in the separation and identification of lipids. Silicic acid is used
as the adsorbent on a glass plate or on a glass slide and offers the advantage of simplicity, ease of manipulation, sensitivity, rapidity, and high resolving power. For following elution profiles, microscope slides were utilized while for separation of the chromaffin granule membrane lipids and for quantitatively determining the purity of phospholipids, the high resolution two-dimensional TLC technique was used.

2.6.1 Micro-slide TLC

Materials: Adsorbent; silica gel G suspended in chloroform at a concentration of 50 g/100 ml.
Plain microscope slides (2.5 x 7.5 cm.)

TLC microslides were prepared by dipping the microscope slides into a slurry of silica gel G and allowing the thin film of silica to air dry. Prior to runs, the silica was heat activated for approximately 30 sec. over a hot plate and the lipid sample was applied with a capillary tube. The TLC plate was run either in an acid or a base solvent. The acid solvent consisted of chloroform-methanol-glacial acetic acid-water system (25/15/4/2) and the base system consisted of a chloroform-methanol-ammonia-water mixture (90/54/5.7/5.4). After each run, the solvent was evaporated by gently heating the slide over a hot-plate and subsequently sprayed with the appropriate stain for detecting specific phospholipids (discussed in section 2.6.3).

2.6.2 Two-Dimensional Thin Layer Chromatography (2D-TLC)

In order to separate the complex mixture of phospholipids in the
chromaffin granule membrane or to determine the purity of the phospholipids quantitatively, use was made of 2D-TLC according to the method of Broekhuyse (65). Pre-coated TLC plates (Silica Gel 60, 0.25 mm. thickness, 20 × 20 cm. plates) were used. They were activated at 120°C in the oven for one hour before use. After sample application, the plate was run vertically in the base solvent system, dried and subsequently run horizontally in the acid solvent system. The composition of the solvent systems are described in section 2.6.1. After the runs, the plate was dried and exposed to iodine vapour for visualization of the phospholipids. The amounts of specific phospholipids components was determined by scraping off the silica corresponding to the specific phospholipid species and determining the amount of phosphorus by the method of Fiske and Subarrow (66). Figure 6 shows the separation of the chromaffin granule membrane lipids achieved by 2D-TLC.

2.6.3 Phospholipid Spray Reagents for Identification

Phosphorus reagent

The specific reagent for detection of phospholipids is the molybdenum blue reagent. Reagent I was made up by adding 40.11 g of MoO$_3$ to 1 L of 25 N H$_2$SO$_4$, and boiling gently until the molybdic anhydride was dissolved. Reagent II was prepared by adding 1.78 g of powdered molybdenum to 500 ml of Reagent I and boiling gently for 15 min. The solution was cooled and decanted. The specific phospholipid reagent was prepared by mixing equal volumes of Reagent I and Reagent
Figure 6. Chromaffin Granule Membrane Phospholipids separated by two-dimensional thin layer chromatography. PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; LPC, lysophosphatidylcholine; SPH, sphingomyelin.
II and diluting the solution with 2 volumes of water. This final reagent, molybdenum blue reagent, has a greenish yellow colour and is stable for months.

**Ninhydrin Reagent**

The ninhydrin reagent was used to detect phospholipids containing free amino groups (e.g. phosphatidylethanolamine, phosphatidylserine, and their lyso derivatives). The reagent consisted of 0.2% ninhydrin in butanol saturated with water. When sprayed with ninhydrin, lipids with a free amino group showed up as red-violet spots.

### 2.7 Phosphorus Assay

The amount of phospholipids was determined according to the Fiske and Subbarow method (66). Reagent I was 70% Perchloric Acid, Reagent II contained 0.22% ammonium molybdate made up in 2% (v/v) concentrated $\text{H}_2\text{SO}_4$, and Reagent III was made up by dissolving 30 g of Sodium Bisulfite, 1 g of sodium sulfite, and 0.5 g 1-amino-2-napthol-4-sulfuric acid at 40°C and was filtered after storing it overnight in the dark in order to remove the crystals.

The procedure for detecting the amount of phosphorus was as follows. Between 0.1 and 0.5 umol of phospholipid sample was hydrolyzed by addition of 0.5 ml. of perchloric acid and one hour digestion at 196°C. In order to prevent evaporation, the tubes were covered with glass marbles. When the hydrolysis was complete (i.e. solution was clear),
Figure 7. Phosphorus standard curve
the tubes were cooled and 14 ml of ammonium molybdate reagent was added followed by 0.6 ml of the Fiske-Subarrow reagent and immediate mixing. Colour was allowed to develop by heating the tubes in a boiling water-bath for 15 min. The tubes were cooled and absorbance at 830 nm. was recorded. Figure 7 show the standard phosphorus curve (0-0.5 umol P).

2.8 **Preparation of Phospholipid vesicles**

The phospholipid model systems were obtained by mixing appropriate quantities of lipid in chloroform and then evaporating the chloroform under a stream of nitrogen. The thin film of phospholipids was then stored under vacuum for 2 hr. in order to remove any residual solvent. The lipid was subsequently hydrated in the buffered sucrose solution and sonicated intermittently (30 sec intervals) in an ice-water bath employing a tip sonicator. Sonication was continued until the dispersion became optically clear (approximately 5 min).

2.9 **$^{31}$P-NMR Experiments**

All $^{31}$P-NMR experiments were performed on a Bruker WP 200 NMR Spectrometer, operating at 81 MHz for phosphorus, which was equipped with proton decoupling and temperature control. Accumulated free induction decays were obtained for up to 2,000 transients employing a 11 μsec. pulse and 0.8 sec interpulse time.
2.9.1 Bilayer to Hexagonal $H_{II}$ Transition Temperature

In order to determine the bilayer to hexagonal transition temperature of phosphatidylethanolamines, $^{31}$P-NMR spectra were accumulated over the appropriate temperature range by employing the VART (variation temperature) program. Briefly, the phospholipid sample (50 μmol) was transferred to an NMR tube and the chloroform was evaporated under a stream of nitrogen. Subsequently, the tube was stored under vacuum for 2 hr and the lipid was hydrated in 1.0 ml of NMR buffer (100 mM NaCl/10 mM HEPES/1 mM EDTA/10 % $D_2O$, pH 7.4) by vortexing. $^{31}$P-NMR spectra at various temperatures were accumulated and the temperature of the bilayer to hexagonal transition was determined. Figure 8 and 9 show the spectra obtained for the bilayer to hexagonal $H_{II}$ transition for soya and egg PE respectively. Their transition temperatures were 15° and 30° C respectively.

2.9.2 Polymorphic Behaviour of the Chromaffin Granule Membrane and Extracted Lipid Liposomes

The chromaffin granule membranes and their total lipids were isolated as described in sections 2.2 and 2.5.1 respectively. The $^{31}$P-NMR experiments on the chromaffin granule membrane were performed by packing the membranes into an NMR tube containing buffered sucrose with 10 % $D_2O$. The extracted membrane phospholipids were viewed after evaporating the chloroform under a stream of nitrogen, storage under
Figure 8. Bilayer to Hexagonal $H_{II}$ transition of Soya Phosphatidyl-ethanolamine.
Figure 9. Bilayer to Hexagonal $H_{II}$ transition of Egg Phosphatidylycerol-ethanolamine.
vacuum for 2 hr., and subsequent hydration with 1.0 ml. of NMR buffer. Addition of calcium from a stock 1 M or 0.1 M solution of CaCl₂ was immediately followed by vortexing.

2.9.3 **Quantification of the amount of Chromaffin Granule Membrane Phospholipids contributing to the \( ^{31}P\)-NMR signal**

In order to quantify the amount of chromaffin granule membrane phospholipids contributing to the \( ^{31}P\)-NMR signal observed, two types of calibration experiments were performed. Initially, the ratios, \( \frac{R_{CG}}{R_{PC}} \), of the integrated signal intensities (recorded sequentially under exactly similar experimental conditions) from a standard sample of egg yolk phosphatidylcholine and the chromaffin granule membrane sample were obtained. This was subsequently compared first, to the ratio obtained from phospholipid phosphorus assays of the standard egg PC sample and the granule membrane sample. Secondly, \( \frac{R_{CG}}{R_{PC}} \) was compared to the ratios of the egg PC standard and the chromaffin granule membrane \( ^{31}P\)-NMR signal intensities after the addition of 0.4 ml. Triton X-100 to both samples. This was sufficient detergent to solubilize both membrane systems, giving rise to translucent dispersions and a narrow, symmetric \( ^{31}P\)-NMR spectrum. In the case of the ratios obtained from phospholipid phosphorus assays and after solubilization with detergent, it may be presumed that all the chromaffin granule phospholipids contribute to the ratio \( \frac{R_{CG}}{R_{PC}} \) obtained. Therefore, comparison of the ratios obtained employing the intact granule membrane to those obtained via
phospholipid phosphorus assay and after detergent treatment gave a measure of the amount of phospholipid not detected in the intact granule membrane by $^{31}$P-NMR.

2.9.4 Influence of Exogenous Lipids on the Chromaffin Granule

$^{31}$P-NMR techniques were employed to investigate the influence of exogenous lipids on the chromaffin granules in the presence and absence of Ca$^{2+}$. The following protocol was followed. Intact chromaffin granules (large granule fraction) were incubated at 37°C for 15 min. either in the presence or absence of exogenous PE-PS (3:1) vesicles with and without the addition of calcium. These incubated preparations were subsequently concentrated for $^{31}$P-NMR studies by centrifugation at 17,000 g for 15 min. The packed pellet was washed with buffered sucrose by gently swirling and then transferred into a NMR tube for signal accumulation. Since the chromaffin granules contain high concentrations of ATP, the dominant features of the $^{31}$P-NMR spectra were the three peaks corresponding to the $\alpha$, $\beta$, and $\gamma$ phosphates of ATP and one peak corresponding to inorganic phosphate. Thus, release of the chromaffin granule contents was monitored by the presence or absence of the ATP peaks. The major disadvantage of this assay was the large amount of chromaffin granules required for each experiment (approximately 100 mg protein). Therefore, a much more economical spectrophotometric assay was employed during majority of the experiments.
2.10 **Spectrophotometric Release Assay**

The spectrophotometric release assay varied from the \(^{31}\text{P-NMR}\) experiments in that the contents released by the chromaffin granules were measured rather than the contents remaining inside the granules. Typically, chromaffin granules (0.8-1.2 mg protein, 40-50 \(\mu\)l of the large granule fraction) in buffered sucrose were incubated in the presence of sonicated vesicles, CaCl\(_2\), or NaCl at 25°C for 15 min in a total volume of 1.0 ml. Where CaCl\(_2\) was not added, a corresponding milliosmols of NaCl was added in order to minimize release due to osmotic differences. The reaction was stopped by addition of 4.0 ml ice-cold buffered sucrose and the chromaffin granules were pelleted at 17,000 g for 10 min. The supernatant was assayed for the release of the chromaffin granule contents, namely protein, ATP, and catecholamines. A reproducible level of about 25% background release was observed in all controls in agreement with the observations of Hillarp et al (67). Release of contents was hence expressed as a percentage after subtracting the background. A measure of the total release of the chromaffin granule contents was taken as the amount release after lysing the chromaffin granules in 5.0 ml of 5 mM HEPES, pH 7.0, followed by a freeze-thaw cycle.

2.11 **Assaying for the release products of the Chromaffin Granules**

**Protein**

The amount of protein released in the supernatant was measured
by the procedure of Lowry et al (58). The determination was made in 1.0 ml of the supernatant and the blank contained 1.0 ml of buffered sucrose.

**Catecholamines**

The amount of catecholamines was measured according to the procedure of Von Euler and Hamberg (68). Briefly, to 1.0 ml of the supernatant, 1.0 ml of 1 M Acetate buffer (pH 6.0), 50 µl of 10 % SDS, and 0.2 ml of 1 N iodine solution was added. After exactly 10 min., 0.2 ml of 0.5 M sodium thiosulfate was added to bleach the colour of the oxidized catecholamines and absorbance at 530 nm was read immediately.

**Total Contents**

Since the contents of the chromaffin granules absorb quite strongly at 265 nm, a very convenient assay for monitoring release was simply to determine the absorbance of the supernatant at 265 nm. It was found that the results obtained by this assay correlated very favourably to those obtained by the protein and catecholamine determinations (see figure 10). Therefore, in the majority of the release experiments, the A$_{265}$ assay was employed.
Figure 10. Correlation of chromaffin granule release products. The amount of release of chromaffin granule contents in the presence of exogenous PE-PS (3:1) vesicles with subsequent addition of CaCl$_2$ as determined by absorbance at 265 nm.($\Delta$), amount of protein (○), and catecholamine content (□) in the supernatant.
Chapter III: RESULTS

3.1 Structural Organization of the Chromaffin Granule Membrane

The first priority in approaching the exocytosis process involving the chromaffin granule and the chromaffin cell plasma membrane was to establish the dynamic structural organization of the endogenous lipids of the chromaffin granule membrane. As indicated in the introduction, $^{31}$P-NMR is a useful technique for determining the polymorphic preferences of phospholipids in model and biological membranes.

3.1.1 Chromaffin Granule Membrane and Total Extracted Lipid

The $^{31}$P-NMR spectra arising from the isolated chromaffin granule membranes, as well as model liposomal systems consisting of hydrated preparations of the total extracted lipids are indicated in figure 11. Two features are apparent. First at $37^\circ$C, a large majority of the endogenous granule membrane lipids exhibit a $^{31}$P-NMR lineshape characteristic of the bilayer phase. This is with the exception of a small component ($\leq 5\%$ of the total phospholipid) which gives a signal of isotropic motional averaging. This may arise from small membrane fragments or lipids in other structures which undergo isotropic averaging (see figure 3). Secondly, the model membrane liposomal system of the total extracted chromaffin granule membrane lipids (figure 11c) exhibits a very similar bilayer spectrum. This observation is consistent with the structural role of phospholipids in an intact membrane,
Figure 11. 31P-NMR spectra of chromaffin granule membrane and chromaffin granule membrane systems. 81.0 MHz 31P-NMR spectra at 37°C arising from (a) isolated chromaffin granule membranes; (b) isolated chromaffin granule membrane in the presence of 10 mM CaCl₂; (c) liposomes composed of lipids extracted from chromaffin granule membrane and (d) as (c) in the presence of 10 mM CaCl₂. All preparations contained 10 mM Tris–HAc (pH = 7.2) and, where CaCl₂ was not present, 2 mM EDTA. The solutions used for the biological membrane contained 10% D₂O, whereas that of the liposomes contained 90% D₂O. 0 ppm refers to the resonance position of sonicated phosphatidylcholine vesicles.
that is maintenance of the bilayer integrity.

Since calcium ions have been found to be essential for the exocytosis process, it was of interest to study its effect on the membrane and the membrane model systems of the chromaffin granule. Figure 11 shows that the addition of 10 mM CaCl₂ results in little change in the biological membrane spectrum (figure 11b) and in the appearance of a relatively small component (≤ 10% of that total phospholipid), possibly arising from phospholipids in the hexagonal $H_{II}$ phase in the model system composed of the isolated total lipids (figure 11d).

3.1.2 Contribution of Endogenous Phospholipid to $^{31}$P-NMR Signal

In order to show what fraction of the endogenous chromaffin granule membrane phospholipids was actually detected, the observed intensity of the $^{31}$P-NMR signal for the granule membranes was quantitatively calibrated against a known amount of egg PC liposomes as described in the methods section. Figure 12 shows the $^{31}$P-NMR spectra of the egg PC liposomes and chromaffin granule membrane taken under similar parameters and figure 12b shows the spectra taken after solubilization of the phospholipids with Triton X-100 detergent. The ratios of the chromaffin granule membrane phospholipid signal intensity to that of egg PC standard, $R_{PC}^{CG}$, were 0.35 for the intact systems, 0.36 as determined by phospholipid phosphorus assays, and 0.37 as determined after solubilization with Triton X-100. These results indicate that more
Figure 12. Quantitative calibration of chromaffin granule membrane phospholipids detected by $^{31}$P-NMR. The amount of chromaffin granule membrane phospholipids detected by $^{31}$P-NMR signal was determined as described in the methods section. Spectra (a) are the signals observed for the standard Soya PC sample and the chromaffin granule membrane obtained under similar conditions. Spectra (b) are the signals observed after the membranes were solubilized by Triton X-100. It was assumed that all the phospholipids contributed to the $^{31}$P-NMR signal upon solubilization.
than 90% of the endogenous chromaffin granule phospholipids contribute to the observed $^{31}\text{P-}\text{NMR}$ signal.

3.2 Influence of Exogenous Lipids

The second stage of the research was to investigate the influence of exogenous lipid and calcium on chromaffin granule release. This was first approached by $^{31}\text{P-}\text{NMR}$ techniques and subsequently by spectrophotometric techniques.

3.2.1 $^{31}\text{P-}\text{NMR}$ Experiments

As indicated in the methods section, intact chromaffin granules (large granule fraction) were incubated in the presence or absence of exogenous lipid vesicles with the subsequent addition of $\text{Ca}^{2+}$ and then concentrated by centrifugation for $^{31}\text{P-}\text{NMR}$ studies. Figure 13 shows the spectra obtained after incubation of intact chromaffin granules at $37^\circ\text{C}$ in the absence (fig. 13a) and in the presence (fig. 13b) of 10 mM $\text{Ca}^{2+}$. In both cases, the dominant features of $^{31}\text{P-}\text{NMR}$ spectra arise from the three phosphate groups of ATP and that of inorganic phosphate. These spectra confirmed previously published spectra of intact chromaffin granules (69). The presence of ATP in the spectrum of fig. 13b indicated that the concentration of calcium of up to 10 mM did not induce significant release of the granules contents. This observation was in agreement with similar electron-microscopic studies.
of intact chromaffin granules done by Edwards et al (70). Against this background, the results were quite dramatic when the granules were incubated in the presence of varying amounts of PE-PS (3:1) vesicles (10 min., 37 C) to which calcium was subsequently added and incubation continued for an additional 5 min. These results are illustrated in figure 13c-e. Defining R as the molar ratio of added exogenous phospholipid vesicles to endogenous chromaffin granule lipid, it is clear that for R=6 (fig. 13e) complete release of ATP has occurred as revealed by the absence of ATP in the chromaffin granules. In addition, a new spectral component is apparent in the region of -7 ppm. which coincides with the position of the low field peak arising from phospholipids in the hexagonal H_{II} phase (53).

The appearance of the hexagonal H_{II} phase phospholipid was not unexpected on the basis of the behaviour of the PE-PS (3:1) in the presence of calcium (fig. 14). The addition of calcium to PE-PS vesicles induces immediate precipitation of the vesicle suspension and triggers the formation of the hexagonal H_{II} phase as indicated by the characteristic $^{31}$P-NMR lineshape in figure 14b. This result was consistent with previous studies done on bilayer liposomal PE-PS systems (29) where the presence of calcium also triggered precipitation and hexagonal H_{II} phase formation.

3.2.2 Spectrophotometric Release Assays: Soya PE-PS

In order to further characterize the ability of exogenous lipid
Figure 13. $^{31}$P-NMR spectra of chromaffin granules in the presence of calcium and in the presence of exogenous soya PE-PS (3:1) vesicles and calcium. 81.0 MHz $^{31}$P-NMR spectra at 37°C obtained from (a) intact chromaffin granules; (b) granules incubated at 37°C for 15 min in the presence of 10 mM CaCl$_2$; (c) same as (a); (d) chromaffin granules incubated in the presence of PE-PS vesicles (R = 4) for 10 min. at 37°C, followed by introduction of CaCl$_2$ to a concentration of 10 mM, after which the incubation at 37°C was continued for an additional 5 min; (e) same as (d) with the exception that R = 6. R is defined as the molar ratio of exogenous (vesicular) PE-PS phospholipid to endogenous (chromaffin granule) phospholipid. The buffered sucrose medium (see methods) was employed throughout. For other details see Methods section.
Figure 14. $^{31}$P-NMR spectra of Soya PE-PS (3:1) vesicles.

81.0 MHz $^{31}$P-NMR spectra at 25 C arising from (a) sonicated PE-PS vesicles; (b) sonicated PE-PS vesicles to which 5 mM CaCl$_2$ was added. The buffered sucrose medium containing 10 % D$_2$O was employed.
to act as adjuncts for $\text{Ca}^{2+}$-stimulated release of granule contents, an independent and a more economical spectrophotometric assay was performed as summarized in the methods section. The results of $^{31}$P-NMR experiments using the PE-PS model systems were confirmed by the spectrophotometric assay. Figure 15 illustrates the percentage of chromaffin granule contents released in the presence of varying amounts of exogenous PE-PS vesicles with subsequent addition of 5 mM $\text{Ca}^{2+}$. At a concentration corresponding to a model membrane phospholipid to chromaffin granule membrane phospholipid ratio, $R$, of 5 (mol/mol) there was complete release of chromaffin granule contents when $\text{Ca}^{2+}$ was present. This contrasted strongly with the results obtained when PE-PS vesicles alone were added as well as the situation when $\text{Ca}^{2+}$ alone was present, in which cases no release above the control level was observed. Also, no significant release for calcium concentrations as high as 10 mM was observed. Therefore, these results clearly establish a requirement for both PE-PS vesicles and $\text{Ca}^{2+}$ as indicated by $^{31}$P-NMR results. Furthermore, the amount of release was also sensitive to the order in which these agents were added to the chromaffin granules prior to incubation. In figure 15 when $\text{Ca}^{2+}$ was present prior to the addition of PE-PS vesicles, the release was significantly lower. This behaviour was interpreted as arising from $\text{Ca}^{2+}$ induced precipitation of the vesicles to form H_{II} phase aggregates before the exogenous vesicles could interact with the chromaffin granules.
Figure 15. Release of chromaffin granule contents in the presence of Ca$^{2+}$ and exogenous Soya PE-PS (3:1) vesicles as assayed by spectrophotometric techniques:

- (■) incubation in the presence of PE-PS vesicles (15 min.) where 5 mM CaCl$_2$ was added after 10 min. incubation;
- (●) incubation in the presence of PE-PS vesicles where 5 mM CaCl$_2$ was introduced prior to vesicle addition. R is the molar ratio of exogenous lipid to chromaffin granule phospholipid.
The mechanism by which PE-PS vesicles act as adjuncts for Ca\(^{2+}\)-stimulated release of chromaffin granule contents is of particular interest. It may imply that the release resulted from Ca\(^{2+}\) induced fusion of the vesicles with the granule membrane followed by lysis as a result of the fusion event itself, or by the presence of non-bilayer lipid in the granule membrane which would no longer support the bilayer structure. As indicated by Tilcock and Cullis (29) the addition of Ca\(^{2+}\) to PE-PS systems results in a structural segregation of the PS component into crystalline (presumably "cochleate") regions, allowing the PE to revert to the \(H_{III}\) phase it prefers in isolation. Questions then arise whether it was the ability of Ca\(^{2+}\) to induce crystalline cochleate structures or the hexagonal phase organization (or both) which was related to the lytic event.

These questions were approached by testing the ability of vesicles composed of specific phospholipids which would preferentially adopt a bilayer or a non-bilayer conformation under the incubation conditions. The phospholipid systems utilized were egg PE-PS, pure PS, and pure cardiolipin (CL).

3.2.3 Egg PE-PS vesicles

As mentioned previously, the bilayer to hexagonal \(H_{III}\) transition temperature of pure egg PE was 25°C. Therefore the pure egg PE would adopt a bilayer conformation at 20°C and a hexagonal \(H_{III}\) conformation
Figure 16. Release of chromaffin granule contents in the presence of soya and egg PE-PS (3:1) vesicles at 20°C and 35°C: (□) incubation in the presence of soya PE-PS vesicles at 20°C and 35°C with subsequent addition of 5 mM CaCl₂; incubation in the presence of egg PE-PS vesicles and 5 mM CaCl₂ at 20°C (○) and at 35°C (△).
at 35°C. The results obtained after incubating egg PE-PS (3:1) mixtures at 20°C and 35°C are shown in figure 16. It is evident that significant release of the chromaffin granule contents occurred when the egg PE-PS vesicles were incubated at 35°C. As a control incubations with soya PE-PS vesicles resulted in significant release at both temperatures. This experiment strongly suggests a possible role for the occurrence of non-bilayer hexagonal $H_{II}$ phases in Ca$^{2+}$-stimulated release of the chromaffin granules as one of the parameters that was influenced at the two temperatures was the bilayer to hexagonal $H_{II}$ transition.

3.2.4 Pure PS vesicles and pure CL vesicles

Calcium induces formation of crystalline cochleate structures for PS dispersions (71,72) while the addition of calcium to cardiolipin model systems triggers formation of the hexagonal $H_{II}$ phase (26). Since it was of interest to ascertain which of the two phases promoted release of the chromaffin granule contents, pure PS and pure CL vesicles were tested as adjuncts for Ca$^{2+}$-stimulated release. As shown in figure 17 both PS and CL vesicles were effective adjuncts for Ca$^{2+}$-stimulated release of chromaffin granule contents. These results therefore did not clearly distinguish the preferred conformations necessary for release.

3.2.5 PC-PS vesicles

Phosphatidylcholine is a bilayer lipid and therefore does not
Figure 17. Release of chromaffin granule contents in the presence of various exogenous lipid vesicles in the presence of Ca\(^{2+}\): (○) incubation in the presence of pure soya PS vesicles; (■) incubation in the presence of pure CL vesicles; (▲) incubation in the presence of soya PC-PS (3:1) vesicles. R is the molar ratio of exogenous lipid to chromaffin granule phospholipid.
undergo bilayer to nonbilayer phase transitions in isolation or in the presence of calcium. Addition of Ca\(^{2+}\) to PC-PS will only result in the interaction of Ca\(^{2+}\) with the acidic PS molecules causing lateral phase separation of the two lipids into separate domains. This observation has recently been observed by freeze-fracture studies of Jacobson and Papahajoupoulos (73). Therefore, the interaction of PC-PS vesicles with chromaffin granules was investigated.

The results in figure 17 indicate that no significant release of chromaffin granule contents resulted from incubation of PC-PS (3:1) vesicles with the chromaffin granules. Identical results were obtained when equimolar PC-PS (1:1) mixtures were employed. These observations imply that the occurrence of a hexagonal \(H_{\text{II}}\) structure may be an important factor in promoting Ca\(^{2+}\)-stimulated release.

3.2.6 Erythrocyte Lipid Systems

Finally, it was of interest to extend the above observations to model systems which may approximate the composition of the inner monolayer of the chromaffin cell plasma membrane more closely. One of the best characterized plasma membranes is that of the red blood cell. Since its asymmetry with respect to phospholipids, proteins, and carbohydrates has been extensively investigated, it was a convenient model system for study. The polymorphic preferences of the inner and outer erythrocyte monolayers had also been recently investigated by Hope.
Figure 18. Release of chromaffin granule contents after incubation with erythrocyte lipid vesicles: (□) incubation in the presence of outer monolayer lipids (15 min.) where 5 mM CaCl\(_2\) was introduced after 10 min.; (●) incubation in the presence of inner monolayer lipids (15 min.) where 5 mM CaCl\(_2\) was introduced after 10 min. R is the molar ratio of exogenous lipid to chromaffin granule phospholipid.
and Cullis (60). It was shown that the inner monolayer model systems partially adopted the hexagonal H$_{II}$ phase in the presence of Ca$^{2+}$, whereas the outer monolayer systems did not.

The ability of model systems composed of human erythrocyte phospholipids in proportions corresponding to the composition of the inner and outer monolayers to act as adjuncts for Ca$^{2+}$-stimulated release of chromaffin granules was investigated. Figure 18 shows that the inner monolayer model systems acted as effective adjuncts for release of granule contents whereas the outer monolayer model systems did not. Larger ratios of exogenous (vesicular) inner monolayer phospholipid to endogenous (granule) phospholipid were required than for the other adjunct systems discussed before. This situation has been tentatively attributed to the instability of the sonicated inner monolayer systems, which tended to aggregate shortly after sonication, as indicated by an increasingly cloudy lipid dispersion.

3.2.7 **Calcium Titration**

In all the above experiments, excess Ca$^{2+}$ (5 mM) was used so that the limiting component was the amount of phospholipid vesicles. When the amount of Ca$^{2+}$ was made limiting, it was found that at least 2 mM Ca$^{2+}$ was required for causing effective release of the chromaffin granule contents (see fig. 19) in the presence of excess PE-PS vesicles (R = 4). This value corresponded with the concentration of Ca$^{2+}$ req-
Figure 19. Effect of calcium concentration on the release of chromaffin granule contents in the presence of excess Soya PE-PS (3:1) vesicles (R = 4) for 15 min. where CaCl$_2$ was added after 10 min.
quired to induce the formation of the hexagonal $H_{II}$ phase in analogous PE-PS liposomal systems (29).
Chapter IV: DISCUSSION

4.1 Models of Membrane Fusion

A brief discussion of the models proposed for membrane fusion over the past decade would be useful for illustrating some of the concepts for the membrane fusion event and also for the interpretation of the results in this thesis. Four popular models have been proposed, namely, the lysolecithin model, the fluidity model, the crystallization model, and the polymorphic model.

4.1.1 Lysolecithin and Membrane Fusion

Rubin (74), Guttler and Clausen (75) suggested the formation of lysophosphatides in promoting membrane fusion and experimental support was presented a few years later by Lucy and coworkers (76) when they fused hen erythrocytes in vitro with lysolecithin. They also observed that the cells were very unstable and lysed within a period of 30 seconds. Studies with artificial lipid membranes have indicated that the insertion of a wedge-shaped molecule such as lysolecithin could promote substantial perturbation in the packing of the lipid molecules (77). Also, due to the highly lytic properties of lysolecithin, its production during membrane fusion would need to be confined to highly localized sites so that the integrity of the rest of the membrane would be maintained. Cells have been found to possess
enzymes responsible for the conversion of lysolecithin to non-lytic derivatives (78) but the levels of enzymes responsible to generate lysolecithin and its removal appear to vary significantly between different cells and even between different membranes of the same cell. It has also been proposed that the high concentrations of lysolecithin found in the chromaffin granules (79) and mast cell granules (80), both of which fuse with the plasma membrane, may be involved in the exocytosis process.

Although it is possible that membrane fusion occurring in vivo may involve bilayer to micellar transitions, no experimental support for this concept has been presented. Also, the fact that the turnover of lysolecithin in the adrenal medulla is not altered during secretion (81) argues against the participation of lysolecithin. Hence, the role of lysolecithin in mediating fusion is only supported by evidence of fusion under experimental conditions and similar fusion events have also been demonstrated by other lypophilic and lipolytic agents which create a similar type of disordering in the membrane. Such agents include retinol, oleic acid, glycerol mono-oleate, propyleneglycol, and phospholipase C. Also, studies of virus-induced cell fusion have failed to provide evidence for the involvement of lysolecithin. Therefore, the role of lysolecithin in membrane fusion is as yet unclear.

An important contribution of this hypothesis was that disordering of the fusing membrane seemed to be a common feature of the fusion
4.1.2 **Fluidity and Membrane Fusion**

The significance of redistribution of intramembranous particles (presumably representing integral proteins) during virus-induced fusion (82) and in other *in vivo* fusion events including that of the chromaffin granules (35,83) and mast cell granule (36) secretion has led to suggestions that the redistribution and aggregation of intramembranous particles (IMP) in fusing membranes provide areas devoid of proteins (35,36). Therefore, fusion is restricted to areas of particle-free membranes and solely involves the interaction between the phospholipids of the two opposing membranes. This hypothesis was extended by Ahkong et al. (84) by the suggestion that the redistribution of the particles would increase membrane fluidity. But, no direct experimental evidence has been presented except from the studies of model membrane systems which indicate that prior to fusion, the phospholipids have to be in a fluid liquid-crystalline state (101). Furthermore, fusogenic compounds such as lysolecithin and myristic acid have been observed to decrease fluidity while dimethylsulfoxide (DMSO) has been shown to increase fluidity (85). Therefore, the exact role of fluidity in promoting membrane fusion is as yet unclear.

4.1.3 **Role of Calcium**

Calcium has been found to be essential for fusion in a wide variety
of fusion events in both natural and model systems (102). In natural fusion systems, Ca$^{2+}$ has been demonstrated to induce processes such as exocytosis in the chromaffin cells (52), mast cells (86), and in other neurosecretory cells (87), by either introducing calcium directly into the cell or by calcium inophores A23187 and X537A. Also, the exocytotic process has been inhibited by blocking calcium entry into the cell with lanthanum, Mg$^{2+}$, Co$^{2+}$, and D-600 (2,88). In model systems composed of acidic phospholipids, fusion has been observed only in the presence of calcium.

Although the exact mechanism by which calcium promotes fusion is unknown, the strong interaction between calcium and acidic phospholipids has led to two recent models of membrane fusion.

4.1.4 Crystallization Model of Fusion

The interaction of calcium with phospholipids, particularly acidic phospholipids, has been extensively investigated by Papahadjopoulos and coworkers (89,101). Calcium has been shown to affect the permeability properties, form two to one (PS/Ca$^{2+}$) complexes, cause crystallization of the acyl chains of acidic phospholipids and thereby raising their gel-liquid crystalline transition temperature, and causing phase separations in mixtures of acidic and neutral phospholipid model systems (PS/PC). On the basis of such observations Papahadjopoulos has proposed a dual role for Ca$^{2+}$ in mediating fusion.
Firstly, the presence of Ca\(^{2+}\) is suggested to promote close apposition of adjacent membranes by enhancing electrostatic interaction between them (90) and by forming a specific intermembrane complex (91). Secondly, Ca\(^{2+}\) induces destabilization of the apposed membranes by formation of crystalline domains of acidic phospholipids which would represent sites at which fusion would occur (90).

Support for such a mechanism of membrane fusion has been shown in the case of pure PS model systems but no evidence has thus far been presented for an in vivo situation or in situations where biological membranes have been employed.

4.1.5 Polymorphic Model of Fusion

The observations that specific phospholipids in a biological membrane can adopt a non-bilayer structure in isolation (22) and that Ca\(^{2+}\) can trigger such structures in mixed model membranes (29) has led to the proposal of a polymorphic model of membrane fusion. The main precept of such a model is that at some stage in the fusion event, whether it is mediated by protein or lipid, a portion of the lipids must experience a departure from the bilayer structure.

Studies with erythrocyte ghost membranes by Hope and Cullis (28), where incorporation of fusogens, oleic acid or glycerol mono-oleate, into the membranes at concentrations sufficient to induce cell fusion have been shown to produce a bilayer to hexagonal H\(_{\II}\) phase trans-
ition in some portion of the membrane phospholipids. Verkleij et al. (53) have also presented freeze-fracture evidence from model membrane systems showing the occurrence of intramembranous lipidic particles. It was suggested that these IMP (presumably inverted lipidic micelles) were possible intermediaries in the bilayer to hexagonal \( H_{II} \) transitions (95). Very recently, Hope and Cullis (28) have shown a positive correlation between formation of a non-bilayer phase and the extent of cell fusion in human erythrocyte ghosts. On the basis of these observations, a molecular model of membrane fusion was proposed where the intermediate structure consisted of hexagonal \( H_{II} \) phase lipid cylinders or inverted micelles (28,30,98).

The suggestion of inverted micelles in the fusion process has also been presented by other independent workers. For example, Lau and Chan (92), studying alamethacin induced fusion by proton NMR, Pinto da Silva (93), studying freeze-fracture results of the fusion of peripheral vesicles with plasmalemma of zoospores of the fungus, Phtophthora palmivora, and Gingell and Ginsberg (94), from a theoretical standpoint, have all proposed similar intermediate structures. Although the polymorphic fusion model has been supported by experimental evidence from model membrane systems, it has as yet been difficult to provide evidence from an \textit{in vivo} situation due to limitations of available biochemical techniques.
4.2 Chromaffin Granule Membrane

The $^{31}$P-NMR results obtained from the isolated granule membrane and model systems composed of the extracted lipid clearly established that the large majority (90% or more) of the endogenous phospholipid experienced the bilayer phase at physiological temperature. This was consistent with the role of the lipid component which is primarily structural in nature, that is, serving to maintain granule integrity. It should be noted that while this behaviour appeared self-consistent and was similar to results obtained from other biological membranes, such as the erythrocyte ghost membranes (21,30), certain observations for organelle membranes indicate that such behaviour cannot be assumed a priori. In the case of rat liver endoplasmic reticulum, for example, two laboratories have independently reported the occurrence of isotropic motional averaging for endogenous phospholipids (96,97). The observation that calcium did not induce major changes in the motional behaviour of isolated granule membrane phospholipids was consistent with the observed inability of calcium to increase release of intact granule contents above background levels.

4.3 Phospholipids as Adjuncts for $Ca^{2+}$-Stimulated Release of Chromaffin Granules

The major result of this thesis concerns the observation that lipid vesicles which undergo structural transformations in the presence of $Ca^{2+}$ could act as adjuncts for $Ca^{2+}$-stimulated release of chromaffin
granule contents. It is reasonable to suppose that this ability is associated with Ca\(^{2+}\)-induced fusion of the vesicles with the granule and that lysis resulted either during the fusion event or as a result of the presence of non-bilayer lipid in the granule membrane. In fact, it is difficult to imagine how the vesicles could produce such dramatic effects without interacting directly with the chromaffin granule membrane.

The common property of the phospholipid vesicles exhibiting the lysis ability was that Ca\(^{2+}\) induced aggregation and formation of larger structures for the vesicle system in isolation. In the case of the PE-PS system, Ca\(^{2+}\) induces lateral phase separation of the PS molecules which results in the fusion between the vesicles and formation of large hexagonal H\(_{II}\) structures by the PE molecules. In contrast, addition of Ca\(^{2+}\) to the EC-PS systems simply causes lateral phase separation but no fusion due to the retention of the bilayer configurations by both lipids. As for pure cardiolipin and phosphatidylserine systems, addition of Ca\(^{2+}\) results in the formation of large structures of hexagonal H\(_{II}\) cylinders and cochleate formation respectively. The observation that both systems acted as efficient adjuncts for release suggested that the detailed nature of these large structures was not a determining factor. Therefore, the major factor contributing to their ability to act as adjuncts was that both cardiolipin and phosphatidylserine in the presence of Ca\(^{2+}\) become destabilized and must fuse between themselves or with
any membrane that they might be interacting with. Hence, in the process of embedding themselves into the granule membrane, the integrity of the chromaffin granule membrane is disrupted and the granule contents released.

The relationship between the above results and $Ca^{2+}$-stimulated release of the chromaffin granule contents \textit{in vivo} is not completely obvious. However, if the inner monolayer lipid composition of the chromaffin cell plasma membrane approximates that of the human erythrocyte plasma membrane, it would imply that the presence of calcium would destabilize the inner monolayer in the sense that a sizable fraction of the phospholipids would prefer the hexagonal $H_{II}$ phase. As has been indicated elsewhere (99), formation of the $H_{II}$ phase from previously bilayer systems appear to proceed as an \textit{inter}-bilayer event. Thus, lipids in the outer monolayer of the chromaffin granule membranes closely apposed to destabilized regions of the inner monolayer of the plasma membrane could serve to relieve this instability by forming local short cylinders ($H_{II}$ structures) or inverted micelles (lipidic particles) by combining with the inner monolayer phospholipids of the chromaffin cell plasma membrane. This type of reasoning seems consistent with the results obtained when the inner and outer erythrocyte model systems were employed as adjuncts. Release of the chromaffin granule contents was only observed in the presence of inner monolayer model systems which are unstable in the presence of calcium.
4.4 Possible Implications for the Mechanism of Exocytosis

From the results of this thesis, certain speculations can be made suggesting an _in vivo_ mechanism of exocytosis. The role of calcium in the fusion event could be first to aid close apposition between the chromaffin granule and the plasma membrane, and second to induce lateral phase separation of the bilayer stabilizing phospholipids (particularly acidic phospholipids such as PS) which would result in local domains of non-bilayer lipids forming _inter_-bilayer lipidic micellar structures. A model depicting such a mechanism of exocytosis with the lipidic particle as the intermediary step is illustrated in figure 20.

As mentioned previously, the inverted micelles have been implicated as intermediaries in fusion processes occurring in Ca$^{2+}$ - induced fusion of model lipid systems of PC-CL vesicles (99) and in the temperature induced fusion of PE-PC-cholesterol vesicles (95). The observation that fusion regions _in vivo_ appear to be devoid of membrane particles as detected by freeze-fracture is potentially in disagreement with the intermediary role of lipidic particles in fusion _in vitro_ (35,36). However, recent observations on the mechanism of release from mast cells (100) suggest that intramembrane particles are present at or near the fusion site.
Figure 20. Proposed mechanism of exocytotic release of chromaffin granule contents in vivo. PM refers to the chromaffin cell plasma membrane and CG denotes the chromaffin granule.
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